A molecular, morphological and biological characterisation of the genus *Globodera* (Nematoda: Heteroderidae) in South Africa

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Declaration

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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Abstract

A molecular, morphological and biological characterisation of the genus *Globodera* (Nematoda: Heteroderidae) in South Africa is presented. The aims of the study were to determine the spread of the genus in South Africa; to study the systematics and describe the characteristics of the group and to gain a more complete understanding of the biology of the group as agricultural pests. Surveys were conducted in the Cape Floristic Region and in all the potato-producing areas of South Africa. The surveys unearthed new species of cyst nematodes and determined the spread of *Globodera rostochiensis* in the country. Phylogenetic analysis of sequences from ITS-rDNA was used to infer phylogenetic relationships among cyst nematodes from South Africa. The analyses established the distinct phylogenetic positions of cyst nematode populations from South Africa relative to an array of other cyst nematode species and indicated the existence of four new species of cyst nematodes. Analysis of random amplified polymorphic DNA (RAPD) banding patterns revealed intraspecific genetic variation amongst populations of *Globodera rostochiensis*. In order to provide molecular protocols for the accurate identification of South African cyst nematodes, species-specific primers and restriction enzymes were tested for their ability to discriminate between local *Globodera* spp. A combination of the molecular, morphological and morphometric characteristics of these populations were used to describe three new species of cyst nematodes. Experiments to determine the effect of storage temperature on the viability and hatching of South African populations of *G. rostochiensis,* showed differences in the responses of different populations to different storage temperatures. Experiments to determine the effect of field conditions on the viability and hatching of South African populations of *G. rostochiensis,* indicated that a decline in viable eggs in cysts from different populations occur, but suggests that the cysts will be able to survive for much longer in these

soils than was expected. Spontaneous hatch was the main contributor to the decline of viability of cysts in the soil. Recording of soil temperatures in different locations indicated that the average temperature at 20 cm depth was approximately 20°C, the optimum temperature for the reproduction of *G. rostochiensis*, as confirmed by *in vitro* reproduction tests, which also showed that multiplication and survival is influenced negatively when the temperatures rise above 25°C. Reproduction on differential potato clones confirmed the pathotype of all the South African populations of *G. rostochiensis* as Ro1. After assessing the reproduction of *G. rostochiensis* on indigenous solanaceous plants, it was concluded that none of these plants induce substantial hatch in *G. rostochiensis*, nor do they support multiplication of the nematode. The results of this project have an impact on inquiries at all taxonomic levels, while also having an essential practical application in nematology. Knowledge of the distribution, pathogenicity, survival potential and reproduction capacity of *Globodera* species in South African soils are valuable for the design of effective management strategies as well as regulatory measures.

Uittreksel

‘n Molekulêre, morfologiese en biologiese karakterisering van die genus *Globodera* (Nematoda: Heteroderidae) in Suid-Afrika word aangebied. Die doelwitte van die studieprojek was om die verspreiding van die genus in Suid-Afrika vas te stel, om die sistematiek van die groep te bestudeer en om ‘n meer volledige begrip van die biologie van die groep te bekom. Opnames is in die Kaapse Floristiese streek en in al die aartappelproduserende gebiede in die land gedoen. Tydens die opnames is nuwe sist nematode spesies gevind en die verspreiding van *Globodera rostochiensis* in Suid-Afrika is vasgestel. Filogenetiese analises van die basispaar opeenvolgings van ITS-rDNS is gebruik om die filogenetiese verwantskappe tussen die sist nematodes van Suid-Afrika vas te stel. Die spesifieke filogenetiese posisies van die nematodes, relatief tot ander spesies het gedui op die teenwoordigheid van 4 nuwe spesies. ‘n Analise van “random amplified polymorphic DNA” (RAPD) bandpatrone het intraspesifieke variasie tussen populasies van *G. rostochiensis* uitgewys. Diagnostiese tegnieke, aan die hand van spesies-spesifieke inleiers en restriksie-ensieme, is geevalueer vir hul vermoë om *Globodera* spesies van mekaar te onderskei. ‘n Kombinasie van molekulére, morfologiese en morfometriese karaktertrekke is gebruik vir die beskrywing van drie nuwe *Globodera* spesies. Eksperimente om die effek van verskillende temperature op die lewenskragtigheid en uitbroei van Suid-Afrikaanse populasies van *G. rostochiensis* vas te stel, het verskille in die reaksies van die poulasies uitgewys. Eksperimente om die effek van veldtoestande op die lewenskragtigheid en uitbroei van Suid-Afrikaanse populasies van *G. rostochiensis* vas te stel, het gewys dat alhoewel ‘n afname in die lewenskragtigheid van eiers plaasvind, sal die siste nog steeds langer in die grond oorleef

as wat verwag is. Spontane uitbroei van eiers was die grootste oorsaak van die afname in lewenskragtigheid van siste in die grond. Die monitoring van grondtemperature in verskillende lokaliteite, het gewys dat die gemiddelde temperatuur, 20 cm onder die grond, nagenoeg 20°C was. Die optimum temperatuur vir die aanteling van *G. rostochiensis,* soos gewys deur *in vitro* toetse is ook 20°C, maar ‘n skerp daling vind plaas by temperature hoer as 25°C. Aanteling op verskillende aartappel cultivars, het gewys dat die patotipe van plaaslike populasies van *G. rostochiensis,* Ro1 is. Toetse op inheemse *Solanum* plante het gewys dat die plante nie goeie gashere vir *G. rostochiensis* is nie. Die bevindings van hierdie studieprojek het ‘n impak op die taksonomie van die groep en kennis van die verspreiding, patogenisiteit en oorlewing van die nematodes onder Suid-Afrikaanse toestande is van waarde vir die daarstelling van effektiewe beheerstrategiee en wetstoepaslike regulasies.

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Chapter 1

Introduction

The cyst-forming plant parasitic nematodes are contained in the subfamily Heteroderinae of the family Heteroderidae (Evans & Rowe, 1998). As the most highly evolved and economically important nematode parasites of plants, they stand out by virtue of their efficient parasitic adaptions (Siddiqi, 2000). They are defined by their capacity to retain eggs inside the female body, which is transformed to a cyst at the completion of the female life cycle (Subbotin *et al.* 2010). The second stage juveniles (J2) remain dormant in the eggs, until hatch is induced by appropriate stimulation, mostly in the form of host-plant root exudates. The eggs and juveniles in the cysts remain viable for a number of years (Siddiqi, 2000). In addition to the formation of cysts, the subfamily is characterised by sedentary feeding and sexual dimorphism. The adult female is globular, with most of the body exposed on the root surface, while the anterior part is buried in the root. Males are vermiform, active and apparently do not feed as migratory adults (Subbotin *et al.* 2010).

The round cyst nematodes, lacking a terminal cone are included in the genus *Globodera.* Evans & Rowe (1998) divides the genus *Globodera* geographically into three main groups: The *Globodera tabacum* group of species from North America, the potato cyst nematodes, *Globodera rostochiensis* (Wollenweber, 1923) Skarbilovich, 1959 and *Globodera pallida* Stone, 1973, from South America and a small group of *Globodera* spp., which are found in the old world and parasitizes the Asteraceae. Subbotin *et al.* (2010) recognise 10 species of *Globodera,* which do not all fit into this grouping (i.e. *Globodera zelandica* Wouts, 1984,

which parasitizes tree fuchsia in New Zealand). *Globodera* is distributed in Europe, North and Central America and Asia (five species each), South America (four species) and Oceania and Africa (each with three species) (Subbotin *et al.* 2010). *G. rostochiensis* and *G. pallida* are reported from all the continents, but are believed to have spread there from South America (Turner & Evans, 1998).

The notorious potato cyst nematodes (PCN) *G. rostochiensis* and *G. pallida*, are among the most heavily regulated nematodes in agricultural commerce. Their success as agricultural pests can be attributed to their adaptability to variable environments and their ability to survive adverse conditions for extended periods of time (Turner & Evans, 1998). *G. rostochiensis* was reported for the first time in South Africa in 1971 (Kleynhans, 1998). After strict quarantine measures were thought to be successful in eradicating the pest at that time, it was reported again, this time in the Western Cape, after an absence of almost 28 years (Knoetze *et al.* 2004). *G. rostochiensis* is listed as a prohibited pest in both the Plant Improvement Act (Act No. 53 of 1976) and Agricultural Pest Act (Act No.36 of 1983). Distribution of PCN by means of seed potatoes is also prevented in the South African Seed Potato Certification Scheme. Potato cyst nematodes have also been recorded from the African continent from Libya, Algeria, Morocco, Tunisia, Egypt, Sierra Leone, Mozambique and South Africa (Kleynhans, 1998), but no indigenous *Globodera* species have been found in Africa, until the discovery of an unknown *Globodera* species in the Sandveld region of South Africa (Knoetze *et al.* 2006).

Consequently, the need has arisen to determine the spread and biodiversity of the group in South Africa. Thus, the present study will include a survey in the potato-producing areas of South Africa for the presence of potato cyst nematodes, as well as a survey in the Cape Floristic Region of South Africa for the presence of indigenous cyst nematodes. To further

characterise the group their systematics and characteristics will be studied by describing and comparing the morphology and taxonomic characteristics of local species and populations found in the surveys. Molecular techniques, such as DNA sequencing will also be utilised to study the phylogenetic relationships of the local species within the group and to assess the intraspecific genetic variation among geographic populations. Molecular techniques, like the polymerase chain reaction and restriction fragment length polymorphisms, will also be used to develop quick, reliable diagnostic methods that will allow growers and regulatory agencies to discriminate between quarantine and non-quarantine nematodes. Furthermore, it has become necessary to gain a more complete understanding of the biology of the group as agricultural pests, enabling nematologists to make informed decisions for the management of these nematodes. In this regard, the effect of local conditions on the survival, hatching and reproduction of South African populations of *G. rostochiensis* has been studied. It was also deemed necessary to determine the pathotypes of South African populations of *G. rostochiensis* and to assess the reproduction of *G. rostochiensis* on indigenous solanaceaous hosts. Finally, since it has been discovered on fallow potato fields in an important seed-producing area, it was important to determine the threat of an indigenous *Globodera* species to the potato industry, by assessing its reproductive ability on potatoes.

To summarize, the aim of the study was to determine the spread of *Globodera* spp. in South Africa; to study the systematics and describe the characteristics of the group in South Africa and to study certain aspects of the biology of the group. The objectives were as follows:

- To undertake surveys to determine the distribution of the nematode and assess the species diversity of the group in South Africa
- Study the phylogenetic relationships of the local species within the group, based on molecular sequence data

- Asses the intraspecific genetic variation among geographic populations
- Develop a quick, reliable diagnostic method that would allow growers and regulatory agencies to discriminate between quarantine and non-quarantine nematodes
- Describe and compare the morphology and taxonomic characteristics of local species and populations
- Study the effect of local conditions on the survival, hatching and reproduction of South African populations of *G. rostochiensis*
- Determine the pathotypes of South African populations of *G. rostochiensis* and
- Asses the reproduction *G. rostochiensis* on indigenous solanaceaous hosts
- Determine the threat of indigenous *Globodera* spp. to the potato industry

The results of this project will have an impact on inquiries at all taxonomic levels, regarding this group of nematodes, while also having an essential practical application in nematology. Knowledge of the distribution, pathogenicity, survival potential and reproduction capacity of *Globodera* species in South African soils will be valuable for the design of effective management strategies as well as regulatory measures.

Literature

EVANS, K. & ROWE, J.A. (1998). Distribution and economic importance. In: Sharma, S.B. (Ed.). *The cyst nematodes*. Dordrecht, The Netherlands, Kluwer Academic Publishers, pp. 1-30.

KLEYNHANS, K.P.N. (1998). Potato cyst nematodes (*Globodera* species) in Africa. In: Marks, R.J. & Brodie, B.B. (Eds.). *Potato Cyst Nematodes. Biology, distribution and control.* Wallingford, UK, CAB International, pp. 347 - 351.

KNOETZE, R., MALAN, A.P. & MOUTON, C. (2006). Differentiation of South African potato cyst nematodes (PCN) by analysis of the rDNA internal transcribed spacer region. *African Plant Protection* 12, 103 – 110.

KNOETZE, R., MALAN, A.P., SWART, A. & PIETERSE, W. (2004). Present status of potato cyst nematode in South Africa (Abst.). *African Plant Protection* 10, 129.

SIDDIQI, M. R. (2000). *Tylenchida parasites of plants and insects*, 2nd edition. Wallingford, UK, CABI Publishing, xvii + 833 pp.

SUBBOTIN, S.A, MUNDO-OCAMPO, M. & BALDWIN, J.G. (2010). *Systematics of cyst nematodes (Nematoda: Heteroderidae).* Nematology monographs and perspectives Volume 8A. (Series editors: Hunt, D.J. & Perry, R.N.). Leiden, The Netherlands, Brill.

TURNER, S.J. & EVANS, K. (1998) The origins, global distribution and biology of potato cyst nematodes (*Globodera rostochiensis* (Woll.) and *Globodera pallida* Stone). In: Marks, R.J. & Brodie, B.B. (Eds.). *Potato Cyst Nematodes. Biology, distribution and control.* Wallingford, UK, CAB International, pp. 7-26.

Chapter 2

A survey of the Cape Floristic Region of South Africa for the presence of indigenous cyst nematodes (Nematoda: Heteroderidae)

Introduction

The genus *Globodera* can be divided geographically into three main groups: The *G. tabacum* group of species from North America, the potato cyst nematodes, *G. rostochiensis* (Woll.) Skarbilovich and *G. pallida* Stone, from South America and a small group of *Globodera* species, which are found in the old world and parasitizes the Asteraceae (Evans & Rowe, 1998). Association by descent can be postulated for *Globodera* spp. from the New World, parasitising Solanaceae and for those from the Eurasian palearctic parasitising Asteraceae, because Solanaceae and Asteraceae are both placed in the highly natural dicotylendon sub-class Asteridae. Stone (1979) suggested that *Globodera* might have originated in Gondwanaland, on the part of the landmass that later became South America. The ancestors of the *Globodera* species parasitizing the Asteraceae in Europe were suggested to have been carried northwards when fragments of Gondwanaland encountered Laurasia (Subbotin *et al.*, 2010), creating an isolated evolutionary niche, where these species might have co-evolved with their hosts (Stone, 1983). Subbotin *et al.* (2011) suggested that the divergence of the two main *Globodera* lineages might have occurred subsequent to the break-up of Africa and South America in the Mid-Cretaceous, because of the association thereof with the time of origin for the Solanaceae. They also argued that the evolution of the Punctoderinae cannot be explained solely by the separation of the continents and diffusion expansion, suggesting a

scenario whereby the ancestral Punctoderinae gave rise to a modern *Globodera* lineage that was introduced to South America or Africa via long distance dispersal from North America.

No indigenous *Globodera* species has been found in Africa until the discovery of such a species in the Sandveld region on the West Coast of South Africa (Knoetze *et al.* 2006), giving rise to new speculations about the origins of the group. The Sandveld is situated in the Cape Floristic Region (CFR) (Low & Rebello, 1996), a biodiversity hotspot of global significance (Myers *et al.*, 2000) and the smallest, but richest plant kingdom in the world. As the discovery of one new *Globodera* species in South Africa suggests the possibility of the discovery and description of more new representatives of *Globodera* from Southern Africa, the author was prompted to initiate a survey of the CFR for the presence of indigenous cyst nematodes. These species may then be a valuable contribution to the study of the evolution and biogeography of the cyst nematode group.

Materials and Methods

THE SURVEY AREA

The CFR, stretches from north of Clanwilliam in the west of South Africa to Port Elizabeth in the East (Fig. 2.2), an area of 87,892 km^2 (Cowling & Heijnis, 2001). Samples were collected throughout the region (Figures 2.1, 2.2), representing 29 different vegetation types (Table 2.1). The majority (60%) of the samples were collected in the Sandveld region (mainly Leipoldtville Sand Fynbos, Lambert's Bay Strandveld and Cederberg Sandstone Fynbos vegetation types), because the earlier discovery of a new cyst nematode species in this region, as well as the fact that Africa was once joined to South America, where the potato cyst

nematodes originate from, led to the belief that the West Coast of South Africa might be a centre of speciation for cyst nematodes in Southern Africa. Large parts of the Sandveld is transformed due to agriculture (mainly potato farming), invasive species and urbanisation. Therefore, pockets of undisturbed natural vegetation were identified in the area and sampled. In addition, samples containing unknown cysts, collected from fallow potato fields in the Swartland and Sandveld areas during the systematic sampling of potato-producing areas of South Africa (Knoetze *et al.*, 2006), were also included in this survey.

Members of the Solanaceae were targeted when sampling, to ascertain if any indigenous cyst nematodes that might pose a threat to the potato industry, exists. This approach led to the sampling of several roadside locations, where members of the Solanaceae were present.

SAMPLING METHODS

A 10 m x 10 m block of indigenous vegetation was identified at each sampling point. Soil was collected in the rhizosphere of at most three of the dominant plant species within each block by means of a small spade or a soil auger, depending on the soil texture. The identities of the plants were recorded by means of photographs and herbarium samples where possible. A GPS (global positioning system) reading of the coordinates at the sampling location was recorded. Samples collected from fallow potato fields, each consisted of 60 individual cores that were distributed over a 4 ha area. Fields of more than 4 ha were subdivided into units of 4 ha or less.

EXTRACTION OF CYSTS

Cysts were extracted with a Seinhorst cyst elutriator (Seinhorst, 1964) and washed through an 840 µm aperture sieve over a 250 µm aperture sieve, collecting into the latter. The content of this sieve was transferred to a piece of filter paper in a funnel and left to dry. The dried debris and cysts were examined using a stereomicroscope.

MORPHOLOGICAL CLASSIFICATION OF CYSTS

Cysts were first classified using a stereo-microscope as round or ovoid cysts (*Globodera* and *Punctodera*) or lemon-shaped cysts (*Heterodera* and *Cactodera*). The identity of the cysts was morphometrically and morphologically determined by preserving the terminal pattern as slide mounts (see Chapter 8) and the J2 larvae were processed for slide mounts (see Chapter 8) or used for the molecular characterisation of the specimens.

MOLECULAR CHARACTERISATION

Individual juveniles were handpicked and placed in a 5 µl drop of 1× PCR reaction buffer (16mM $[NH_4]_2SO_4$, 67 mM Tris-HCl pH 8.8, 0.1% Tween-20) containing 60 µg/ml Proteinase K in a sterile PCR tube. The nematode was then cut into small pieces with a sterile scalpel blade. The tube was then incubated at 60°C for 15 minutes, and a further 5 minutes at 95°C.

Two PCR amplification primers were used to amplify the ITS regions, including the 5.8S ribosomal gene, as well as short parts of the 18S and 28S ribosomal genes. The rDNA1 primer (5'-TTGATTACGTCCCTGCCCTTT-3') and rDNA2 primer (5'-TTTCACTCGCCGTTACTAAGG-3') have been described by Vrain et al. (1992) for amplification of the ITS regions.

PCR products were cleaned up and sequenced by Inqaba Biotechnical Industries (Pty) Ltd. Sequence assembly and editing was performed on the CLC DNA Workbench 6.7.1 (http://www.clcbio.com). Sequences were compared to known sequences in the public databases by means of the Basic Local Alignment Search Tool, or BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi)

Results

Occurrence and viability of cysts

A total of 81 blocks of indigenous vegetation and 17 fallow potato fields were sampled as described. Cysts were detected in 24 of these samples, representing the following vegetation types: Leipoldtville Sand Fynbos (10), Lambert's Bay Strandveld (6), Hopefield Sand Fynbos (2), Graafwater Sandstone Fynbos (1), Olifants Sandstone Fynbos (1), Piketberg Sandstone Fynbos (1), Swartland Granite Renosterveld (1), Agulhas Limestone Fynbos (1) and Gamtoos thicket (1) (Table 2.2). Cysts containing eggs and larvae were encountered in the majority of the positive samples. One sample each from the Swartland and Sandveld areas contained only empty cysts.

MORPHOLOGICAL CLASSIFICATION OF CYSTS

The majority of positive samples contained round cysts, lacking a terminal cone. One sample from the Sandveld area contained spheroid to ovoid cysts with an obtuse terminal cone, whilst another from the Eastern Cape contained lemon-shaped cysts, with a terminal cone. Table 2.2 lists the initial morphological classification and viability of the cysts as observed using a stereomicroscope. Detailed descriptions of the populations are provided in Chapters 8 & 9.

MOLECULAR CHARACTERISATION OF CYSTS

Amplification of ITS1+2 regions of the isolates with primers 18S and 28S yielded a single fragment of approximately 1200 bp. Five isolates from samples collected from fallow potato fields in the Swartland and Sandveld areas were selected for sequencing. The remaining viable populations collected from natural veld were also sequenced. Usable sequences were obtained from all, except WK-26. The BLAST results for the different sequences are shown in Table 2.3. The sequences obtained from the isolate from fallow potato fields were very similar, but they differed significantly from all the other sequences, which were in turn all significantly different from each other. The blast results, as well as in-depth molecular characterisation of the populations in Chapter 4 indicate the possibility of at least four new cyst species occurring in the CFR.

Discussion

This survey constitutes the first survey specifically targeted at the detection of indigenous cyst nematodes in South Africa. Other nematode surveys in indigenous areas of South Africa

include the Swartberg Nature reserve (Marais *et al*., 2003), Nama Karoo and Succulent Karoo (Van den Berg et al. 2003), but they were not specifically targeting cyst nematode species. This survey unearthed at least four potentially new species of cyst nematodes. The description of the morphological characteristics, host relationships and phylogenetic analysis of molecular data of these specimens may prove invaluable for the study of the evolution and biogeography of the group. The CFR displays exceptionally high diversity and endemism of vascular plants and invertebrates. Cape Fynbos cover over 41000 km^2 of the CFR, but other vegetation types like renosterveld, karroid shrubland, various thicket types and forest are also present (Cowling *et al*, 2004). The alpha diversity measurement (the number of species in a single plot of one square kilometre) for Cape Fynbos averages around 65 plant species per km^2 (Cowling *et al*, 2004), which complicates the recognition of specific plant-nematode interactions from soil samples from this region. The determination of the host plants of these cysts therefore proved to be quite difficult due to the proliferation of plant species at each sampling point and further investigation is warranted.

Literature

COWLING, R.M. & HEIJNIS, C.E. (2001). The identification of broad habitat units as biodiversity entities for a systematic conservation planning in the Cape Floristic Region. *South African Journal of Botany* 67, 15–38.

COWLING, R.M., RICHARDSON, D.M. & MUSTART, P.J. (2004). Fynbos. In: Cowling, R.M., Richardson, D.M. & Pierce, S.M. (Eds.) *Vegetation of Southern Africa*. Cambridge University Press, Pp 99-130.

EVANS, K. & ROWE, J.A. (1998). Distribution and economic importance. In: Sharma, S.B. (Ed.). *The cyst nematodes*. Dordrecht, The Netherlands, Kluwer Academic Publishers, pp.1-30.

KNOETZE, R., MALAN, A.P. & MOUTON, C. (2006). Differentiation of South African potato cyst nematodes (PCN) by analysis of the rDNA internal transcribed spacer region. *African Plant Protection* 12, 103 – 110.

LOW, A.B. & REBELO, A.G. (eds.) (1996). *Vegetation of South Africa, Lesotho and Swaziland.* Pretoria, DEAT.

MARAIS, M., VAN DEN BERG, E., SWART, A. & VAN DER WALT, Z. (2004). Plant nematodes of the Swartberg Nature Reserve (Abst.). *African Plant Protection* 10, 139

MYERS, N., MITTERMEIER, R.A., MITTERMEIER, C.G., DA FONSECA, G.A.B. & KENT, J. (2000). Biodiversity hotspots for conservation priorities. *Nature* 403, 853–858.

SEINHORST, J.W. (1964). Methods for the extraction of *Heterodera* cysts from not previously dried soil samples. *Nematologica* 10, 87 – 94.

STONE, A. R. (1979). Co-evolution of nematodes and plants. *Symbolae Botanicae Uppsala* 22, 46-61.

STONE, A. R. (1983). Three approaches to the status of a species complex with a revision of some *Globodera* (Nematoda: Heteroderidae), In Stone, A.R., Platt, H.M. & Khalil, L.F. (Eds.) *Concepts in Nematode Systematics*, Systematics Association Special Volume No 22. London, UK, Academic Press, pp. 221-233.

SUBBOTIN, S.A, CID DEL PRADO, I., MUNDO-OCAMPO, M. & BALDWIN, J.G. (2011). Identification, phylogeny and phylogeography of circumfenestrate cyst nematodes (Nematoda: Heteroderidae) as inferred from analysis of ITS-rDNA. *Nematology* 13, 805-824.

SUBBOTIN, S.A, MUNDO-OCAMPO, M. & BALDWIN, J.G. (2010). *Systematics of cyst nematodes (Nematoda: Heteroderidae).* Nematology monographs and perspectives Volume 8A. (Series editors: Hunt, D.J. & Perry, R.N.). Leiden, The Netherlands, Brill.

VAN DEN BERG, E., MARAIS, M. & TIEDT, L.R. (2004). Criconematidae from the Nama and Succulent Karoo Biomes, South Africa. (Abst.). *African Plant Protection* 10, 141

Table 2.1: Samples collected per vegetation type in the Cape Floristic Region of South Africa.

Vegetation type	Amount of samples
Leipoldtville Sand Fynbos	19
Cederberg Sandstone Fynbos	8
Lambert's Bay Strandveld	7
Overberg Sandstone Fynbos	7
Agulhas Limestone Fynbos	6
Piketberg Sandstone Fynbos	6
Graafwater Sandstone Fynbos	5
Olifants Sandstone Fynbos	4
Tsitsikamma Sandstone Fynbos	4
Hangklip Sand Fynbos	3
South Outeniqua Sandstone Fynbos	3
Albertinia Sand Fynbos	2
Eastern Ruens Shale Renosterveld	2
Gamtoos thicket	2
Greyton Shale Fynbos	2
Hopefield Sand Fynbos	2
Kouga Sandstone Fynbos	2
Langkloof Shale Renosterveld	2
Swartruggens Quartzite Fynbos	2
Bokkeveld Sandstone Fynbos	1
Garden Route Granite Fynbos	1
Garden Route Shale Fynbos	1
Groot Brak Dune Strandveld	1
Potberg Ferricrete Fynbos	1
South Langeberg Sandstone Fynbos	1
Southern Coastal Forest	1
Swartland Granite Renosterveld	1
Swartland Shale Renosterveld	1
Western Ruens Shale Renosterveld	1
Total	81

Table 2.2. Details of sampling locations, associated plant genera and cyst shapes of samples containing nematode cysts, collected in the Cape Floristic Region of South Africa.

Nr.	Vegetation type	Collection month	Description of sampling area	Associated plant genera	Viability	Cyst shape
SP-01	Lambert's Bay Strandveld	Jul-99	Fallow potato field	*Conicosia*	Alive	Spheroid, lacking a terminal cone
SP-02	Leipoldtville Sand Fynbos	Aug-99	Fallow potato field	Not specified	Alive	Spheroid, lacking a terminal cone
SP-03	Graafwater Sandstone Fynbos	Oct-99	Fallow potato field	Not specified	Alive	Spheroid, lacking a terminal cone
SP-04	Hopefield Sand Fynbos	Nov-99	Fallow potato field	Not specified	Alive	Spheroid, lacking a terminal cone
SP-05	Leipoldtville Sand Fynbos	Jan-00	Fallow potato field	Not specified	Alive	Spheroid, lacking a terminal cone
SP-08	Lambert's Bay Strandveld	Mar-00	Fallow potato field	Not specified	Alive	Spheroid, lacking a terminal cone
SP-10	Lambert's Bay Strandveld	Jun-00	Fallow potato field	Not specified	Alive	Spheroid, lacking a terminal cone
SP-13	Leipoldtville Sand Fynbos	Apr-01	Fallow potato field	Not specified	Alive	Spheroid, lacking a terminal cone
SP-14	Hopefield Sand Fynbos	Apr-01	Fallow potato field	Not specified	Alive	Spheroid, lacking a terminal cone
SP-16	Lambert's Bay Strandveld	Mar-02	Fallow potato field	Not specified	Alive	Spheroid, lacking a terminal cone
SP-17	Leipoldtville Sand Fynbos	May-02	Fallow potato field	Not specified	Alive	Spheroid, lacking a terminal cone
SP-19	Leipoldtville Sand Fynbos	Jun-02	Fallow potato field	Not specified	Alive	Spheroid, lacking a terminal cone
SP-22	Lambert's Bay Strandveld	May-03	Fallow potato field	Not specified	Alive	Spheroid, lacking a terminal cone
SP-23	Lambert's Bay Strandveld	May-03	Fallow potato field	Not specified	Alive	Spheroid, lacking a terminal cone
SP-24	Leipoldtville Sand Fynbos	Jan-04	Fallow potato field	Not specified	Alive	Spheroid, lacking a terminal cone
SP-25	Leipoldtville Sand Fynbos	Aug-04	Fallow potato field	*Conicosia, Oncosiphon*	Alive	Spheroid, lacking a terminal cone
SP-28	Leipoldtville Sand Fynbos	Mar-07	Fallow potato field	Not specified	Alive	Spheroid, lacking a terminal cone
WK-01	Leipoldtville Sand Fynbos	May-10	Natural vegetation	*Indogifera, Wiborgia, Lampranthus*	Alive	Spheroid, lacking a terminal cone
WK-02	Leipoldtville Sand Fynbos	May-10	Natural vegetation	*Lycium, Solanum, Asparagus*	Alive	Spheroid to ovoid with an obtuse terminal cone
WK-07	Olifants Sandstone Fynbos	May-10	Natural vegetation	*Euryops, Stoebe, Elytropappus*	Dead	Spheroid, lacking a terminal cone
WK-11	Swartland Granite Renosterveld	May-10	Natural vegetation	*Lycium, Eriocephalus*	Dead	Spheroid, lacking a terminal cone
WK-26	Piketberg Sandstone Fynbos	May-10	Natural vegetation	*Euryops, Stoebe*	Alive	Spheroid, lacking a terminal cone
SK-18	Agulhas Limestone Fynbos	Jul-10	Natural vegetation	*Solanum, Chrysanthemoides*	Alive	Spheroid, lacking a terminal cone
OK-14	Gamtoos thicket	Aug-11	Road shoulder	*Nicotiana*	Alive	Lemon-shaped, with a terminal cone

Table 2.3. BLAST results and morphological classification of cyst nematodes, collected in the Cape Floristic Region of South Africa. Only alignments with coverage above 90% were considered.

Isolate	Morphological classification	Top 5 similar species		
		Description	Accession number	Maximum Identity (%)
SP-01	Spheroid, lacking a terminal cone	*Globodera artemisiae*	EU855121	93
		Globodera rostochiensis	GQ294519	93
		Globodera pallida	GQ355975	93
		Globodera tabacum tabacum	GQ294525	93
		Globodera mexicana	EU006709	92
SP-04	Spheroid, lacking a terminal cone	*Globodera artemisiae*	EU855121	93
		Globodera rostochiensis	GQ294519	93
		Globodera pallida	GQ355975	93
		Globodera tabacum tabacum	GQ294525	93
		Globodera mexicana	EU006709	92
SP-13	Spheroid, lacking a terminal cone	*Globodera artemisiae*	EU855121	93
		Globodera rostochiensis	GQ294519	93
		Globodera pallida	GQ355975	93
		Globodera tabacum tabacum	GQ294525	93
		Globodera mexicana	EU006709	92
SP-25	Spheroid, lacking a terminal cone	*Globodera artemisiae*	EU855121	93
		Globodera rostochiensis	GQ294519	93
		Globodera pallida	GQ355975	93
		Globodera tabacum tabacum	GQ294525	93
		Globodera mexicana	EU006709	92
SP-28	Spheroid, lacking a terminal cone	*Globodera artemisiae*	EU855121	93
		Globodera rostochiensis	GQ294519	93
		Globodera pallida	GQ355975	93
		Globodera tabacum tabacum	GQ294525	93
		Globodera mexicana	EU006709	92
WK-01	Spheroid, lacking a terminal cone	*Globodera artemisiae*	EU855121	97
		Globodera hypolisi	AB207273	95
		Globodera millefolii	HQ260407	95
		Globodera sp. (Portugal)	AY090884	95
		Globodera rostochiensis	FJ212162	93
WK-02	Spheroid to ovoid with an obtuse terminal cone	*Heterodera bifenestra*	AY569020	95
		Heterodera schachtii	EF611116	95
		Heterodera filipjevi	GU079654	94
		Heterodera glycines	AF216579	92
		Globodera rostochiensis	GQ294514	92
SK-18	Spheroid, lacking a terminal cone	*Globodera artemisiae*	EU855121	96
		Globodera millefolii	AY599498	96
		Globodera hypolisi	AB207273	96
		Globodera sp. (Portugal)	AY090884	95
		Globodera rostochiensis	FJ212162	93
OK-14	Lemon-shaped, with a terminal cone	*Heterodera bifenestra*	AY569020	95
		Heterodera schachtii	EF611107	93
		Heterodera glycines	GU595432	91
		Heterodera betae	EF611122	91
		Heterodera trifolii	AY590283	91

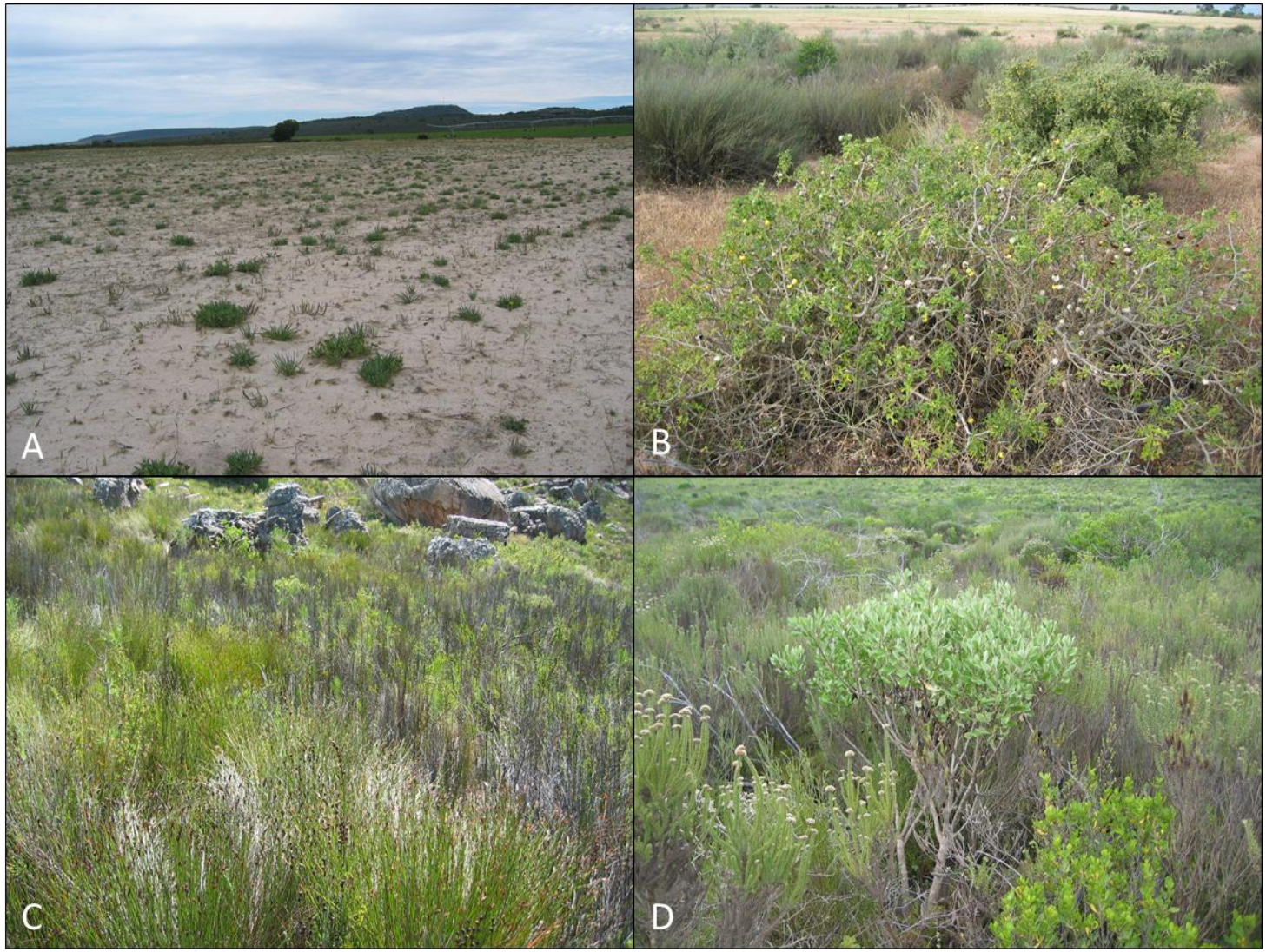

Fig. 2.1. Some of the vegetation types sampled in the Cape Floristic Region of South Africa. A: A Fallow potato field situated in Leipoldtville Sand Fynbos; B: A pocket of Leipoldtville Sand Fynbos adjacent to potato fields; C: Piketberg Sandstone Fynbos; D: Agulhas Limestone Fynbos.

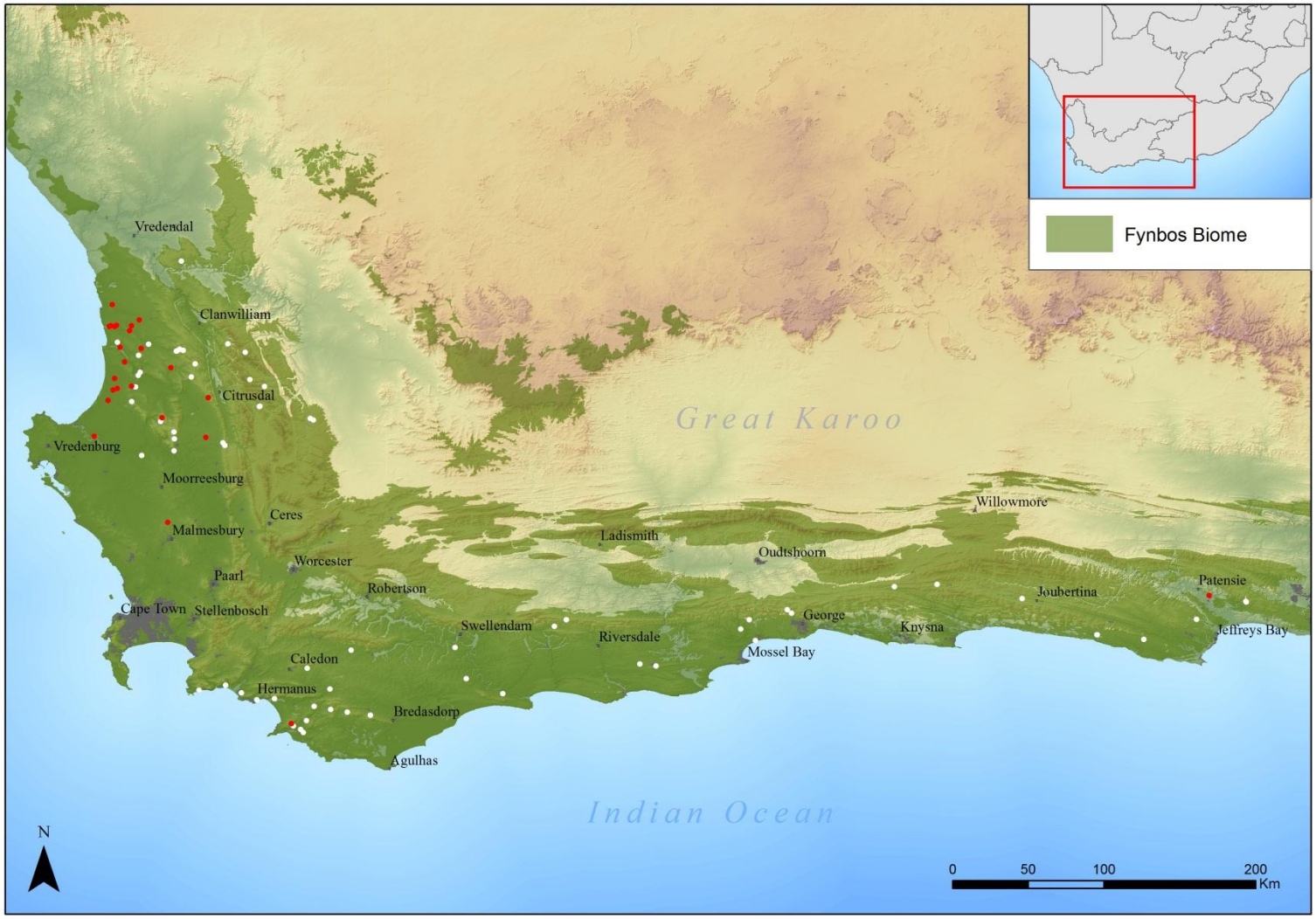

Fig. 2.2. Distribution of samples in the Cape Floristic Region of South Africa. Red icons depict samples that contained cysts. White icons depict samples without cysts. Map produced by the Department of Geography & Environmental Studies, Stellenbosch University.

Chapter 3

A survey to determine the distribution of potato cyst nematodes in the potato-producing areas of South Africa

Introduction

Potential yield losses and the disruption of cropping patterns that would arise from the widespread incidence of potato cyst nematodes (PCN), *G. rostochiensis* (Woll.) Skarbilovich and *G. pallida* Stone, in South Africa are strong reasons why the pest should be contained. Developing and small-scale farmers are especially vulnerable to this nematode because of poor resources and limitations on available land. PCN could also severely affect the industry through quarantine restrictions and/or increased controls. In both the Plant Improvement Act (Act No. 53 of 1976) and Agricultural Pest Act (Act No.36 of 1983), this nematode is listed as a prohibited pest. Distribution of PCN by means of seed potatoes is prevented in the South African Seed Potato Certification Scheme of 15 May 1998, where no tolerance for infection is permissible.

G. rostochiensis was reported for the first time in South Africa in 1971 from an irrigated farm north of Pretoria and subsequently on smallholdings around Johannesburg and Bon Accord (Kleynhans, 1998). Very strict quarantine measures were imposed to prevent the spread of this nematode to other potato producing areas. These measures were successful, allowing the quarantine restrictions to be lifted at that time. During 1999, almost 28 years later, it was reported for the first time in the Western Cape, in the Ceres area (Knoetze *et al.* 2004).

Before the commencement of a countrywide survey in November 2005, 31 plots from 17 farms, representing a total of 464 ha have been placed under quarantine because of the presence of *G. rostochiensis*. These plots were situated in the Ceres (14 plots from three farms) and Sandveld (17 plots from 14 farms) areas. Two new infestations of *G. rostochiensis* were also reported from the Eastern Cape (Gamtoos valley) and Gauteng (Randfontein area) before the commencement of a survey.

Existing PCN policies seek to avoid large-scale future problems. One solution, addressed in this study, is to develop a protocol to manage the spread of the pest, taking into account the following objectives: i) to ensure that traded seed in South Africa remains free of PCN; ii) to isolate infested lands to ensure further spread is prevented; iii) to plant only non-host crops in infested fields and iv) to contain, slowly reduce and finally eliminate the pest. The protocol for the Regulatory Control and Management of the Golden Cyst Nematode in South Africa states that seed potatoes are only to be planted on units not under a served order and tested free of *G. rostochiensis*. A served order will only be lifted after 8 years of non-host cultivation and only if an official test shows it to be free from *G. rostochiensis*. Final certification of the seed is also subject to a negative test result at harvest of the crop. Plantings for table potatoes on infested plots may take place whenever an official test shows the unit to be free of viable cysts.

To be able to further assess the distribution of PCN in South Africa, a countrywide survey needed to be undertaken by the Directorate: Agricultural Products Inspection Services (APIS) of the Department of Agriculture, Forestry and Fisheries (DAFF). Such a survey could lead to the establishment of pest free areas, act as an early warning system to detect the spread of *G. rostochiensis* to new production areas and detect the possible introduction of *G. pallida* into the country.

Materials and methods

PERCENTAGE OF AREAS TO BE SAMPLED

Potatoes are produced in all the provinces of South Africa. Statistically, it is not advisable to predetermine the sampling frequency for representative sampling if the expected frequency of occurrence of the nematode is not known. It is therefore advisable to do sampling according to the maximum capacity of personnel allocated for this task. Taking time and manpower resources into consideration it was recommended that the survey should include approximately 10% of production fields in each area.

Table 3.1 show the recommended amount of samples per province when 10% of the production fields are to be sampled and the prescribed sampling protocol is followed (see 2.4). The original amount of samples per area was calculated proportionally from the estimated areas under potato production in each area during the 2005 season. This information is available on the website of Potatoes South Africa (www.potatoes.co.za).

DELIMITING SURVEYS

G. rostochiensis was already known to occur in the Ceres, Sandveld, Gamtoos and Gauteng areas, which means that these areas had to be subjected to delimiting surveys by APIS. In reality, the production of potatoes takes place in separate production areas within certain provinces (for example: the Sandveld and Ceres areas in the Western Cape and the Gamtoos valley in the Eastern Cape). Therefore it was recommended that the delimiting surveys should

take place in these areas and not the whole province. Following the discovery of the pest in 1999, an operational plan was put into action to determine the extent of the spread of the nematode. This included confirmation of initial reports from the Ceres area, monitoring of all plots registered for export and registered plots for certification of seed potatoes in the Sandveld and Ceres areas. In 2002 a survey of table potato fields in the Ceres area was undertaken. Although the nationwide survey only started in 2005, the data from these surveys will be considered for inclusion in the delimiting survey of the potato-producing areas of the Western Cape Province.

SAMPLING METHODS

Sampling was done according to prescribed methods by the responsible inspectors from APIS in the region. The sampling procedures instructed inspectors to collect one sample consisting of 60 individual cores from each 4 ha area. Whenever plots of more than 4 ha were sampled, they had to be subdivided in units of 4 ha or less. Relevant information regarding variety, location and size of the plots sampled were recorded on a sample form. This included a GPS (global positioning system) reading of the coordinates at the sampling location. Instruction was given that sampling should be distributed over as many farms as possible in an area. Sampling of more than one 4 ha area on the same farm was discouraged. All samples were sent to the Plant Health Diagnostic Services laboratory in Stellenbosch for analysis.

ANALYSIS OF SAMPLES

Cysts were extracted with a Seinhorst cyst elutriator (Seinhorst, 1964). Cysts were washed through an 840 µm aperture sieve over a 250 µm aperture sieve, collecting into the latter. The content of this sieve was transferred to a piece of filter paper in a funnel and left to dry. The dried debris and cysts were examined using a stereomicroscope. Initial identification of the cysts was done using the polymerase chain reaction (PCR) and restriction enzyme digest of the ITS regions as described in Knoetze *et al.* (2006). The identity of cysts, determined as *G. rostochiensis* by PCR, was morphologically confirmed by the Biosystematics Division of the Plant Protection Research Institute (Agricultural Research Council), Pretoria.

Results and discussion

HECTARES SAMPLED PER AREA

Soon after the commencement of the survey in 2005, it became evident that the capacity of the Inspection Services would be under considerable stress to complete the survey in a relatively short period. Consequently no time limit for the completion of the survey was determined. The survey thus continued for six years until November 2011, at which stage it was decided to discontinue it in its present format until the results of the survey were analysed. The continuation of the survey as well as the quarantine status of the nematode would then be reconsidered.

Table 3.2 shows the amount of hectares surveyed compared to the targets that were set in 2005. The area sampled were calculated only from the amount of samples handed in, since it was accepted that all the inspectors followed the prescribed sampling protocol, which states

that one sample should be collected for every 4 ha. All samples collected since 1999, including those collected for export purposes were considered for inclusion in the survey results. From this data it is evident that the amount of samples received from the Free State and Limpopo provinces represents less than the proposed 10% of hectares under potato production in these provinces, in terms of the determining survey.

Figure 3.1 shows the progression of samples from potato fields analysed for the presence of potato cyst nematodes since 1999 in terms of the amount of hectares they represent. Only samples from the Western Cape, Eastern Cape, Free State (for export purposes) and KwaZulu-Natal (for export purposes) were analysed before the commencement of the nationwide survey in 2005. In provinces like the North West and Northern Cape, sampling only began as late as 2007.

A number of countries have implemented survey programs for the detection of potato cyst nematodes. Recent examples are: New Zealand, Australia (Marshall, 1998), Hungary (Elekes-Kaminszky, *et al.*, 2008) and USA (USDA APHIS, 2009). In New Zealand, 25% of seed potato-producing properties were surveyed each year. Priority was given to high risk properties, but all properties were surveyed in a 5-year period. Ten percent of table potatoes were surveyed in regions where PCN had not been found previously, but on high-risk land all potato crops were surveyed annually. However, in New Zealand sampling was carried out predominantly by pre-harvest examination of plant roots and not by soil-sampling. In Victoria, Australia 100% of seed potato crops and approximately 16% of table potatoes were surveyed in 1992. In addition to soil sampling and pre-harvest examination of plant roots, samples were also taken from soil under grading machines. Following the discovery of *G. pallida* in Idaho, the USDA recommended the implementation of a national survey plan for the detection of PCN. The survey plan was designed to sample 25% of the annual commercial

seed potato acreage and 1% of commercial potato production acreage. The EU PCN Directive also requires each member state to complete an annual survey of their ware potato production land to obtain data on the levels and species of PCN present. The directive requires 0.5% of the ware growing area to be sampled annually (Anon, 2011). In South Africa, roughly 40% of potato-producing areas (including seed potatoes) were tested over a period of 12 years (since 1999) or an average of 3.3% per year. It was only in the Sandveld and Ceres regions that testing of all seed-potato plots was compulsory in this period. Therefore, the intensity of the Australasian survey programmes was not quite reached, but the South African effort roughly conformed to the American example, and exceeded the European example, although the latter was designed for a region where the nematode is known to be widespread and are thus aimed at obtaining data on the levels of PCN in the region.

However, by specifying P = 0.02 (2 in 100 plots) as a tolerably low level of infection (based on the actual percentage of infected plots known to us at this stage), and A = 0.01 (1 in 100) as a tolerably low risk of failure to detect such a level, the requisite number of 4 ha units to be sampled can provisionally be determined by n = (log0.02)/log(1-0.01) = 389 units. A different picture emerges for the different provinces (see table 3.3) if this number is corrected for population size.

It can thus be assumed that it is only in the provinces of KwaZulu-Natal, North-West and the Western Cape that the risk of 1 in 100 of not detecting a 2% infection was reached. If the risk of failure (A) to detect the level of infestation is to be increased to 0.03, only two provinces (Limpopo and North-West) would then have failed the test of not detecting a 2% infection (Table 3.3).

DELIMITING SURVEYS

Before the commencement of the nationwide survey, it was already known that the Ceres, Sandveld, Gamtoos and Gauteng production areas needed to be subjected to delimiting surveys. The investigation of a possible infestation in the Philippi area (South-Western Cape) also led to the confirmation thereof and subsequent survey in 2008. Table 3.4 shows the data from delimiting surveys in these areas.

The International Standard for Phytosanitary Measures (ISPM 6) defines a delimiting survey as a "…survey conducted to establish the boundaries of an area considered to be infested by or free from a pest." The manner in which sites are selected is important in the design of delimiting surveys and is also considered as the main difference between delimiting surveys and the other surveys. With delimiting surveys, the initial detection site should be used as a starting point to determine how the pest arrived, where it originated and to where it might have spread. Because the site where a pest is first detected might not be the initial site of the infestation, a delimiting survey can be used to identify the original source of the pest, but it can also be used to trace the pest's possible spread, thereby locating areas that might be infested and will need to be surveyed. The results of a delimiting survey will often have consequences for quarantine and trade and may lead to the establishment of a quarantine or pest free area.

SAMPLING METHODS

When considering the effectiveness of soil sampling procedures, the following factors can be taken into consideration:

- Increasing the number of probes taken per sampled area above 50 cores does not significantly increase detection efficiency (Turner, 1993).
- The sampling pattern may indeed be an important determinant of the success in detecting nematode infestations and the zigzag plan should be adopted in preference to the perimeter plan, as it represents as much of the field as possible, and increases the chance of detecting infestations over a range of levels of aggregation (Perry, 1996).
- When the bulk field sample is small enough for direct examination in the laboratory, sub sampling errors are avoided, but accuracy is sacrificed in reducing the size of the field sample (Southey, 1986).
- The depth to which samples are taken in the top 20 cm of field soils have little or no effect on detection of PCN populations, because the cysts are distributed vertically in a random fashion during cultivation (Boag and Neilson, 1994).
- Generally, one sample should be taken from each area for which a separate decision is to be made, irrespective of the size of the area, but up to maximum of the usual field size (e.g. 4 ha) in a country (Anon, 1991).
- Subdividing large areas into smaller units will go some way to overcome the effects of within field aggregation (Southey, 1986).
- After representative samples have been taken, it is extremely important to protect them from drying, exposure to heat or cold, rough handling and anaerobic conditions (Shurtleff & Averre, 2000)

Taking these factors and resource limitations into consideration, a sampling procedure should be designed which will maximize sensitivity, but avoid sub-sampling errors and ensuring consistency in the probability to detect a given infestation. The current sampling protocol

meets these requirements, but a review of this document is required to ensure adherence to specific details.

Furtermore, if the provinces are regarded as our universum, the farms will be the primary sampling units, which will then be divided in 4 ha units or secondary sampling units. To investigate the characteristics of the universum the following sampling strategies will have to be considered:

- 5 secondaries from each of 10 primaries [Total area = (4 ha)(5)(10) =200 ha] or;
- 1 secondary from each of 50 primaries [Total area = (4 ha)(1)(50) =200 ha]

The preferred sampling strategy is the second example, because the variation among secondaries from different primaries will here be greater than the variation among secondaries from the same primary. This is the reason why sampling of more than one 4 ha area on the same farm was discouraged. However, because samples taken for export purposes were also considered for the survey (because of limited manpower and resources), this principle could not always be adhered to and on occasions numerous samples were received from the same farm.

PLOTS PLACED UNDER QUARANTINE FOR THE PRESENCE OF *G. ROSTOCHIENSIS*

Because *G. rostochiensis* is listed as a prohibited pest in Agricultural Pest Act (Act No.36 of 1983), plots infested with this nematode are placed under quarantine by means of a served order. A served order will only be lifted after 8 years of non-host cultivation and only if an official test shows it to be free from *G. rostochiensis*. Table 3.5 contains a summary of plots

that have been placed under quarantine for the presence of *G. rostochiensis.* To this date, 60 plots from 39 farms, representing a total of approximately 800 hectares have been placed under quarantine because of the presence of *G. rostochiensis*. These plots are situated in the Ceres, Sandveld, Gamtoos, Gauteng and South Western Cape areas. Quarantine has been lifted on 13 of these plots, representing approximately 245 hectares. It is evident that although some of the plots have been quarantined for the required period of 8 years, they have not been re-tested and quarantine regulations have not been lifted.

Recommendations

After the consideration of the results and discussions contained within this document, the following recommendations are made with regard to the future surveying and management of the potato cyst nematodes in South Africa:

- The changing of the pest status of *G. rostochiensis* in South Africa from that of a quarantine pest to a regulated non-quarantine pest as described in ISPM 16 should be considered by the Pest Risk Analysis (PRA) unit of Directorate: Plant Health. In contrast, *G. pallida* or pale cyst nematode should remain a quarantine pest.
- Annual surveying of table potato fields in each province should continue to monitor and contain the spread of the pest to new areas. A survey plan designed to sample 1% of all table potato fields planted each year is recommended. This does not include fields tested for export or certification purposes. Sampling from soil under grading machines should also be investigated.

- In provinces where the pest already occurs, all seed potato plantings should be tested for *G. rostochiensis* before certification.
- A new approach to delimiting surveys to determine how the pest arrived, where it originated and to where it might have spread in order to pinpoint possible infestations should be considered. This approach should urgently be applied to the Gauteng region, since no delimiting survey has been executed there.
- All plots that have been under quarantine for longer than 8 years should be tested and the quarantine lifted as soon as possible, if clean of PCN.

Literature

ANON (1991). Quarantine procedure no. 30. *Globodera pallida* and *G. rostochiensis*: soil sampling methods. *EPPO Bulletin* 21, 233-240.

ANON (2011). Potato Cyst Nematode (PCN) - Official Survey of Ware Potato Production Land. http://thefera.co.uk/plants/plantHealth/pestsDiseases

BOAG, B. & NEILSON, R. (1994). Nematode aggregation and its effect on sampling strategies. *Aspects of Applied Biology* 37, 103-111.

ELEKES-KAMINSZKY, M.; FEKETÉ-PALKOVICS, Á.; TÓTH, Á.; AVAR, K.; BALOGH, ….. (2008). The third nation-wide survey for potato cyst nematodes (*Globodera rostochiensis* (Woll.) and *G. pallida* (Stone) in Hungary (2003-2006). *Növényvédelem* 44 (12), 615-622.

KLEYNHANS, K.P.N. (1998). Potato cyst nematodes (*Globodera* species) in Africa. In: Marks, R.J. & Brodie, B.B. (Eds.). *Potato Cyst Nematodes. Biology, distribution and control.* Wallingford, UK, CAB International, pp. 347-351.

KNOETZE, R., MALAN, A.P. & MOUTON, C. (2006). Differentiation of South African potato cyst nematodes (PCN) by analysis of the rDNA internal transcribed spacer region. *African Plant Protection* 12, 103-110.

KNOETZE, R., MALAN, A.P., SWART, A. & PIETERSE, W. (2004). Present status of potato cyst nematode in South Africa (Abst.). *African Plant Protection* 10, 129.

MARSHALL, J.W. (1998). Potato cyst nematodes (*Globodera* species) in New Zealand and Australia. In: Marks, R.J. & Brodie, B.B. (Eds.). *Potato Cyst Nematodes. Biology, distribution and control.* Wallingford, UK, CAB International, pp. 353 - 394.

PERRY, J.N. (1996). Simulating spatial patterns of counts in agriculture and ecology. *Computers and Electronics in Agriculture* 15, 93-109.

SEINHORST, J.W. (1964). Methods for the extraction of *Heterodera* cysts from not previously dried soil samples. *Nematologica* 10, 87–94.

SHURTLEFF, M.C. & AVERRE, C.W. (2000). *Diagnosing Plant Diseases caused by Nematodes*. Minnesota, USA, The American Phytopathological Society, 317 pp.

SOUTHEY, J.F. (1986). Principles of sampling for nematodes. In: Southey, J.F. (ed.), *Laboratory methods for work with plant and soil nematodes.* London, UK, HMSO, pp. 1-4.

TURNER, S.J. (1993). Soil sampling to detect potato cyst nematodes (PCN) (*Globodera* spp.). *Annals of Applied Biology* 123, 349-357.

USDA APHIS (2009). Pale potato cyst nematode national survey and diagnostic cyst sample forwarding protocols. http://www.aphis.usda.gov/plant_health/plant_pest_info/potato

Table 3.1: Recommended amount of hectares to be sampled per province according to 2005 production statistics.

Province	Area under production (ha)	Recommended sampling area (ha)	Number of samples
Eastern Cape	3577	360	90
FreeState	17954	1796	449
Gauteng	847	88	22
KwaZulu-Natal	3815	384	96
Limpopo	7782	780	195
Mpumalanga	4744	476	119
Northern Cape	1791	180	45
North West	1411	144	36
Western Cape	8376	840	210
Total	50297	5048	1262

Table 3.2: Hectares surveyed per province compared to targets derived from 2005 production statistics

Province	Recommended sampling area (ha)	Area sampled (ha)	Deficit (ha)
Eastern Cape	360	836	0
Free State	1796	1280	516
Gauteng	88	380	0
KwaZulu-Natal	384	1720	0
Limpopo	780	204	580
Mpumalanga	476	504	0
Northern Cape	180	300	0
North West	144	1708	0
Western Cape	840	13100	0

Table 3.3. Four hectare units to be sampled for specified P and A, compared to actual amount of 4 ha units sampled.

Province	Recommended units to be sampled when P = 0.02 and A = 0.01	Recommended units to be sampled when P = 0.02 and A = 0.03	Actual units sampled	Deficit when P = 0.02 and A = 0.01	Deficit when P = 0.02 and A = 0.03
Eastern Cape	271	112	209	62	0
FreeState	358	124	320	38	0
Gauteng	137	80	95	42	0
KwaZulu-Natal	276	113	430	0	0
Limpopo	324	120	51	273	69
Mpumalanga	293	116	126	167	0
Northern Cape	208	100	75	133	25
North West	185	94	427	0	0
Western Cape	328	121	3275	0	0

Table 3.4: Hectares surveyed per production area for delimiting surveys compared to 2005 production statistics.

Province	Production area (ha)	Area sampled (ha)	Positive plots (ha)
Ceres	741	2828	200
Gamtoos	1767	660	232
Gauteng	847	380	21
Sandveld	6442	10016	344
Philippi	502	204	32

Table 3.5: Plots under quarantine for the presence of *Globodera rostochiensis.*

Production area	Plots still under quarantine		Plots where quarantine was lifted	
	Amount	Area (ha)	Amount	Area (ha)
Ceres	17	156	0	0
Sandveld	11	201	13	245
Gamtoos	15	182	0	0
Gauteng	1	4	0	0
Philippi	3	12	0	0
Totals	47	555	13	245

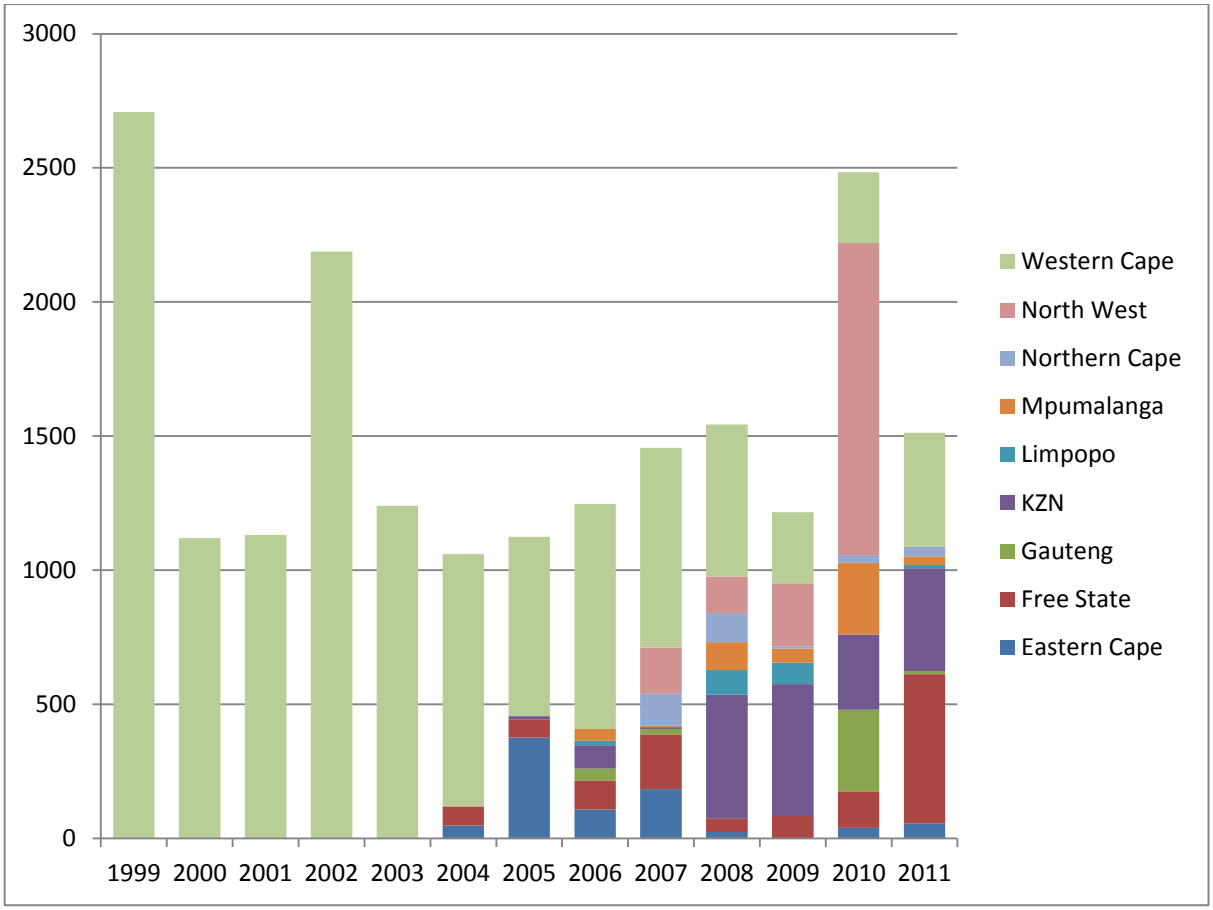

Fig. 3.1. Hectares represented by samples analysed for Potato Cyst Nematodes in a countrywide survey.

Chapter 4

Molecular characterisation and phylogeny of South African populations of cyst nematodes (Nematoda: Heteroderidae) as inferred from analysis of ITS-rDNA

Introduction

The use of molecular data for phylogenetic inference is well established for many groups of organisms. Many authors have suggested that molecular data could be a powerful source of information for the systematics of cyst nematodes (Baldwin, 1992; Bossis & Mugniery, 1993; Ferris & Ferris, 1992; Ferris *et al.*, 1991; Hyman & Powers, 1991 and Powers & Adams, 1993). Inter- and intraspecific genetic variability of species provides proven tools for analysis of the phylogeny of nematodes. Applications of molecular approaches with analysis of ribosomal RNA gene (rRNA) sequences have added new reliability to understanding relationships within cyst nematodes (Subbotin *et al.*, 2011). The ribosomal RNA genes are some of the best characterised gene regions in nematodes (Powers, 2004). The RNA genes are highly conserved, but useful variation is found both in discrete regions of the genes and in the length and sequence of the spacer regions. The ribosomal genes evolve slowly compared to their spacer regions, but still retain some useful genetic information. Typically, the rRNA array consists of three ribosomal genes, 18S, 5.8S, and 28S which are aligned in repeating units, together with their spacer regions, ITS1 and ITS2.

Ribosomal DNA sequence data from five species of *Globodera* were compared by Ferris *et al*. (1995). Phylogenetic analysis of the sequence data indicated that *Globodera pallida*

Stone, 1973 and *G. rostochiensis* (Wollenweber, 1923) Skarbiloviich, 1959 are closely related to undescribed *Globodera* isolates from Mexico, but *G. virginiae* is more distantly related. They stated that their results is consistent with the hypothesis that Mexico is the centre of origin for potato cyst nematodes. Thiery & Mugniery (1996) examined the status of 26 populations of *Globodera* by RFLP's and sequencing of the same region. Four revealed clusters corresponded to *G. rostochiensis*, *G. pallida*, the *G. tabacum* complex and *G. mexicana*, a species of uncertain status. Their data supported the status of *G. mexicana* as a valid species.

Ferris *et al*. (1999) found that sequence data for ITS rDNA for *G. millefolii* and *G. artemisiae* were nearly identical to sequence data for *Cactodera salina* from Mexico. The ITS rDNA sequences of these three species were all about 94% similar to those of two other *Cactodera* species for which ITS rDNA data were obtained. Phylogenetic analysis indicated that, based on the ITS rDNA data, *G. millefolii* and *G. artemisiae* are more closely related phylogenetically to the *Cactodera* species than to other nominal *Globodera* species. The molecular data further suggest that the genus *Cactodera* may comprise two or more morphologically similar but separate groups. The D3 expansion region of the 28S gene and the ITS regions of rDNA sequences from *G. rostochiensis*, *G. pallida*, *G. tabacum tabacum*, *G. tabacum virginiae* and *G. tabacum solanacearum* was aligned and compared by Subbotin *et al* (2000).

Sequence analysis and RFLP's of ITS-PCR products showed that several haplotypes are present in the genomes of *G. rostochiensis* and *G. pallida* populations. Phylogenetic analysis of the ITS sequences obtained from different populations revealed four distinct main clades within *Globodera* parasitising solanaceous plants. They are *G. rostochiensis*, *G. tabacum*, *G. pallida* and an undescribed *Globodera* sp. from South America.

Sequences of the D3 expansion region of 28S rDNA was not useful for the differentiation of *Globodera* spp. Subbotin *et al.* (2001) also sequenced and analysed the ITS regions of ribosomal DNA from 40 taxa of the family Heteroderidae, including the genera *Afenestrata*, *Cactodera*, *Heterodera*, *Globodera*, *Punctodera*, *Meloidodera*, *Cryphodera* and *Thecavermiculatus.* Phylogenetic analyses with maximum-parsimony, minimum-evolution, and maximum-likelihood methods were performed with a range of computer alignments. The cyst-forming nematodes were represented by six main clades corresponding to morphological characters and host specialisation, with certain clades assuming different positions depending on alignment procedure and/or method of phylogenetic inference. Their analyses strongly supported monophyly of the Punctoderinae containing the genera *Cactodera*, *Punctodera*, and *Globodera*. The relatively high level of nucleotide sequence similarity among these three genera suggests that they may have diverged relatively recently and/or in rapid succession from one another. Recognition of a monophyletic genus *Globodera* was not borne out by some of their analyses. They suggested that paraphyly of *Globodera* may simply reflect the general tendency of phylogenetic algorithms to produce unbalanced trees rather than the true evolutionary history of a group with high evolutionary rates. Genetic divergence within this genus reflects the species groupings based on geographical origin and host plants. They are: (i) *G. artemisiae* infecting *Artemisia* in Europe and Asia, (ii) *G. pallida* and *G. rostochiensis* originating from the Andean regions of South America and parasitizing potato and other closely related species of the Solanaceae, and (iii) species of the *G. tabacum* complex being found mainly in the United States and parasitizing other members of the Solanaceae, notably tobacco. They concluded that ITS sequence data are appropriate for studies of relationships within the different species groups and less so for recovery of more ancient speciations within Heteroderidae.

Phylogenetic analysis of ribosomal DNA to infer evolutionary relationships among five species of *Heterodera* collected in Italy and Syria (Sabo *et al.*, 2001) revealed the placement of *H. hordecalis* from Italy, which appears to be only distantly related to other *Heterodera* species in the analysis. The evolutionary relationship based on rDNA data indicates with high bootstrap support a sister taxon relationship to *Cactodera betulae*. They concluded that *C. betulae* and the *H. hordecalis* specimens from Italy both belong to a genus distinct from either *Cactodera* or *Heterodera*.

Sturhan (2002) revised the definition of *Cactodera*, based mainly on an analysis of the host ranges of the species presently placed in the genus, and of selected morphological characteristics. He considered the genus to be monophyletic, after the exclusion of *C. betulae* and *C. johanseni*. *C. betulae* could not be assigned to any of the presently recognised genera of cyst-forming nematodes and therefore a new genus, *Betulodera* was proposed with *B. betulae* as the type and only species.

Phylogenetic analysis by Sabo *et al.* (2002) based on ribosomal DNA sequence data for a previously unknown species of *Globodera* from Portugal, *Punctodera chalcoensis* from Mexico, and *P. punctata* from Estonia showed that the *Globodera* sp. from Portugal, which can be confused with potato cyst nematodes by phytosanitary services when the identification is based only on morphological characters, is clearly different based on molecular data. In addition, the rDNA data show the *Globodera* sp. to be only distantly related to other European *Globodera* species that parasitize Asteraceae. In their analysis, *P. chalcoensis* and *P. punctata* form a sister clade to the *G. pallida* and *G. rostochiensis* clade.

A phylogenetic analysis of the ITS1-rRNA sequence of an unusual cyst nematode from New Zealand, *Paradolichodera tenuissima* shows a close relationship to *Punctodera* and

Globodera (Sturhan *et al.*, 2007). The females weakly tan after death and retain eggs. In a study of relationships among selected cyst-forming and non-cyst-forming species of Heteroderoidea by Ferris *et al.* (2004), one of the non-cyst-forming species, *Ekphymatodera thomasoni*, grouped with cyst-forming species of Heteroderoidea. *Bilobodera flexa*, also a non-cyst-forming species, was separated from all the other taxa by a long branch. *Afenestrata koreana*, with a weakly sclerotized cyst, grouped closely with *H. bifenestra*. They suggested that phylogenetic analyses using molecular data may aid in our understanding of the evolution of cyst formation in nematodes, including the possibility of secondary loss. They also concluded that the usefulness of molecular phylogenetic analyses in nematodes may depend more on the particular selection of taxa than on mere addition of data from additional genes.

Some 134 ITS rRNA gene sequences for circumfenestrate cyst nematodes and two sequences for non-cyst nematodes of the family Heteroderidae, of which 46 were newly obtained, were analysed by Subbotin *et al.* (2011) using phylogenetic and phylogeographic methods. Sequence and phylogenetic analysis combined with known morphological, biological and geographical data allowed the identification, amongst samples original to this study, of several belonging to known valid species as well as others that might be new species. The phylogenetic analysis revealed six major clades for circumfenestrate cyst nematodes: i) *Globodera* from South and North America; ii) *Globodera* from Europe, Asia, Africa and Oceania; iii) *Paradolichodera*; iv) *Punctodera*; v) *Cactodera*; and vi) *Betulodera*. Monophylies of *Punctodera, Cactodera* and *Betulodera* were highly supported. The *Betulodera* clade occupied a basal position on all trees. Phylogeographic analysis suggested a North American origin of Punctoderinae with possible further long distance dispersal to

South America, Africa and other regions. Molecular data supported synonymisation of *G. achilleae* with *G. millefolii* and of *G. hypolysi* with *G. artemisiae*.

The objectives of this study are to analyse the evolutionary relationships among the species of circumfenestrate cyst nematodes occurring in South Africa and to construct a phylogeny of cyst nematodes which includes the South African species.

Materials and Methods

SOURCES OF ISOLATES

Isolates from indigenous populations of cysts were collected in the Cape Floristic Kingdom of South Africa (Chapter 2). The four isolates considered for inclusion in this study are listed in Table 4.1. Since sequences from the cysts collected from fallow potato fields in the Sandveld region were almost identical, (Chapter 2), only one isolate, SP25, was selected for inclusion. As it was morphologically evident that a population of lemon-shaped cysts, with a terminal cone (OK 14), as described in Chapter 2, was not one of the circumfenestrate cyst nematodes, it was not considered for inclusion in this analysis. For ease of reference the isolates will be referred to as SP25, SK18, WK1 and WK2 in this chapter.

PREPARATION OF DNA TEMPLATES

Cysts were cut open and the juveniles removed. Individual juveniles were handpicked and placed in a 10 µl drop of 1× PCR reaction buffer (16mM $[NH_4]_2SO_4$, 67 mM Tris-HCl pH

8.8, 0.1% Tween-20) containing 60 µg/ml Proteinase K in a sterile PCR tube. The nematode was then cut into small pieces with a sterile scalpel blade. The tube was then incubated at 60°C for 15 minutes, and a further 5 minutes at 95°C. The lysate was stored at -80°C until further use.

AMPLIFICATION OF ITS-RDNA

Two PCR amplification primers that amplify the ITS regions, including the 5.8S ribosomal gene, as well as short parts of the 18S and 28S ribosomal genes were used. The rDNA1 primer (5'-TTGATTACGTCCCTGCCCTTT-3') and rDNA2 primer (5'-TTTCACTCGCCGTTACTAAGG-3') have been described by Vrain *et al*. (1992) for amplification of the ITS regions. The primers were synthesized by Integrated DNA Technologies Inc. (Coralville, Iowa, USA). PCR amplifications were carried out using 5 µl of nematode lysate together with 0.5 µM of each primer, dATP, dCTP, dGTP and dTTP each at 200 µM final concentration, 1 × Taq reaction buffer, 1.5 mM $MgCl_2$ and 1 U Taq polymerase. The final reaction volume was 25 µl. The cycling conditions were as follows: denaturation at 94°C for 20 s, annealing at 55°C for 30 s and extension at 72°C for 45 s, repeated for 30 cycles. A 2 min incubation period at 72°C followed the last cycle in order to complete any partially synthesised strands.

DNA SEQUENCING

PCR products of the ITS-rDNA regions were cleaned up and sequenced by Inqaba Biotechnical Industries (Pty) Ltd, using an ABI 3500xL Genetic Analyzer with 24 capillaries. A BigDye version 3.1 dye terminator cycle sequencing kit from Applied Biosystems was used for the reactions. Two internal primers, PunQF1 (5'-GAACTGCAGAAACCTTGA -3') and PunQR1 (5'-TTCACCTACAGCTACCTT -3') were designed and used in addition to the PCR primers 18S and 28S to enable the sequencing of the complete PCR products. Sequence assembly and editing was performed on the CLC DNA Workbench (http://www.clcbio.com).

SEQUENCE ANALYSIS

The ITS-rDNA sequences from the indigenous isolates were aligned with selected sequences of other species from the Heteroderidae (Ferris *et al.*, 1999; Subbotin *et al.*, 2001; Sabo *et al.*, 2002; Tanha Maafi *et al.*, 2003; Manduric & Andersson, 2004; Sirca & Urek, 2004; Uehara *et al.*, 2005; Skantar *et al.*, 2007; Sturhan *et al.*, 2007; Madani *et al.*, 2007; Picard *et al.*, 2008; Yan & Smiley, 2009; Grenier *et al.*, 2010; Madani *et al.*, 2010; Subbotin *et al.*, 2011; Handoo *et al.*, 2012; Lax *et al.*, unpubl.; Moreland & Fleming, unpubl.; Nowaczyk *et al.*, unpubl.) A non-cyst forming heteroderid, *Cryphodera brinkmani* Karssen & Van Aelst, 1999, was used as outgroup species. In order to obtain the best resolution of phylogenetic relationships, sequences were aligned using ClustalX 2.1 with default options (Thompson *et al.*, 1997), MAFFT (Katoh *et al.*, 2005) with the L-INS-i strategy and MUSCLE (Edgar, 2004) with default options. All the available sequences from a clade containing *Globodera* species that parasitise non-solanaceous plants (Subbotin *et al.*, 2011) were included in the alignment, together with selected representatives from the other five major clades of the

circumfenestrate cyst nematodes. Sequences of *Heterodera bifenestra, H. filipjevi* and *H. schachtii* were also included in the alignment, because of the high percentage of identity that were obtained when the sequence from population WK2 were compared to known sequences in the public databases by means of the Basic Local Alignment Search Tool, or BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi), as described in Chapter 2. Sequence alignments were edited to contain only the ITS regions.

Distance analysis of the sequences from indigenous isolates and selected *Globodera* species was conducted in MEGA5 (Tamura *et al.*, 2011). The number of base differences per sequence and the number of base substitutions per site from between sequences were calculated, the latter using the Tamura-Nei model (Tamura & Nei, 1993).

PHYLOGENETIC ANALYSIS

Phylogenetic analyses to resolve the relationships between the indigenous isolates and selected representatives of the Heteroderidae were conducted in MEGA5 (Tamura *et al.*, 2011), using both distance- and character-based methods. The evolutionary history was inferred using the Neighbor-Joining method (NJ) (Saitou & Nei, 1987), whereby the evolutionary distances was computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2004). The alignments was also analysed by using the Maximum Likelihood (ML) method based on the Tamura-Nei model (Tamura & Nei, 1993). In this instance, initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. The Maximum Parsimony method (MP) was also used to obtain a consensus tree using the Subtree-Pruning-Regrafting (SPR) algorithm (Nei & Kumar, 2000), in which the

initial trees were obtained by the random addition of sequences (10 replicates). All ambiguous positions were removed for each sequence pair. The confidence intervals for the various branching patterns in the trees was measured with the bootstrap test (BS) with 1000 replicates (Felsenstein, 1985).

Results

POLYMERASE CHAIN REACTION

Amplification of the ITS regions with primers rDNA1 and rDNA2 yielded a single fragment of 1210 bp, 1213 bp, 1205 bp and 1227 bp for SP25, SK18, WK1 and WK2, respectively.

DNA SEQUENCE CHARACTERISATION

The sequence lengths and frequencies of nucleotide distribution for the different isolates, as well as closely related species, are shown in Table 4.2. Sequences of these isolates were submitted to Genbank (see Table 4.1 for accession numbers). WK2 has the longest ITS1 region (567 bp), but the shortest ITS2 region (197 bp) of the sequences analysed. It also has the highest level of thymine (30%) and lowest level of adenine (20%) of the sequences analysed.

Pairwise distances between closely related *Globodera* species (Table 4.3) show that SP25, SK18 and WK1 differs least from *G. millefolii* (base differences per sequence = 60, 33 and 39, respectively; number of nucleotide substitutions per site = 0.070, 0.037 and 0.045, respectively), and most from *G. mexicana*, *G. ellingtonae* and an undescribed *Globodera*

from Chile (base differences per sequence = 70 - 79; number of nucleotide substitutions per site = 0.083 – 0.092). The sequence obtained from WK2 exhibits a high level of sequence divergence, when compared to the sequences of *Globodera* sp. and other cyst nematode populations analysed here (average base differences per sequence = 241; average number of nucleotide substitutions per site = 0.347). When sequences from only the South African cysts are considered, the greatest difference is between WK1 and SP25 (base differences per sequence = 71; number of nucleotide substitutions per site = 0.084) and the least difference is between WK 1 and SK18 (base differences per sequence = 48; number of nucleotide substitutions per site = 0.055).

PHYLOGENETIC RELATIONSHIPS

Phylogenetic relationships of selected species of the Heteroderidae inferred from ITS-rRNA sequences using Neighbor-Joining (NJ), Maximum Likelihood (ML) and Maximum Parsimony method (MP) are presented in Figures 4.1, 4.2, 4.3. Since minor differences were observed in trees produced with the different alignment programs, trees resulting from the MAFFT (Katoh *et al*., 2005) alignments were used. The resulting trees all consisted of seven major clades with adequate bootstrap support (BS). They are: *i) Globodera* parasitizing non-solanaceous plants (highest BS = 87); *ii) Globodera* parasitizing solanaceous plants (highest BS = 100); *iii) Paradolichodera; iv) Punctodera* (highest BS = 100)*; v) Cactodera* (highest BS = 100), *Heterodera* (highest BS = 96) and *vii) Betulodera*. The position of SP25 in our tree corresponds with the findings of Subbotin *et al*. (2011), occupying a basal position within a lineage of *Globodera* species from Europe, Asia and New Zealand, parasitizing non-Solanaceous plants. In the consensus trees from all the methods, SK18 is included in the

clade of *Globodera* sp. that parasitise non-solanaceous plants, forming a monophyletic group with unidentified *Globodera* spp. from Portugal within that clade (highest BS = 74). WK1 occupies a basal position within a grouping including *G. millefolii*, *G. artemisiae*, SK18 and the unidentified *Globodera* spp. from Portugal (highest BS = 99). However, in the ML tree with the highest log likelihood (Fig 4.3), the roles are reversed i.e. WK1 forming a monophyletic group with unidentified *Globodera* spp. from Portugal, while SK18 occupies the basal position in the larger group. In all the trees, WK2 is included in the *Heterodera* clade, forming a monophyletic group with *H. bifenestra* (highest BS = 99).

Discussion

This study establishes the distinct phylogenetic positions of the cyst nematode isolates from South Africa relative to an array of other cyst nematode species. Characterisation and phylogenetic analyses of sequences from the isolates SP25, SK18 and WK1 confirms their membership of the genus *Globodera*, and supports the hypothesis that they are indeed unique species, previously undescribed. Further investigation into the morphology and morphometrics of these nematodes (see Chapter 8, 9) are necessary to confirm if these nematodes are new species of *Globodera.*

Subbotin *et al.* (2011) speculated that South America or Africa appears to be a centre of origin of *Globodera*, supporting Stone's (1979) hypothesis of a Gondwanaland origin of *Globodera* with subsequent dispersal of the species of this genus to Europe, North America, Asia and Oceania. Subbotin *et al.* (2011) suggested that the divergence of the two main *Globodera* lineages might have occurred subsequent to the break-up of Africa and South America in the Mid-Cretaceous, because of the association thereof with the time of origin for

the Solanaceae. Subbotin *et al.* (2011) also argued that the evolution of the Punctoderinae cannot be explained solely by the separation of the continents and diffusion expansion, suggesting a scenario whereby the ancestral Punctoderinae gave rise to a modern *Globodera* lineage that was introduced to South America or Africa via long distance dispersal from North America.

The discovery of these new *Globodera* species in Southern Africa supports the theory of a Gondwanaland origin of the genus and the theory that divergence of the two main *Globodera* lineages might have occurred subsequent to the break-up of Africa and South America. The ancestors of the *Globodera* species parasitizing the Asteraceae in Europe were suggested to have been carried northwards when fragments of Gondwanaland encountered Laurasia creating an isolated evolutionary niche, where these species might have co-evolved with their hosts (Stone, 1983). Although the host plant of the South African *Globodera* species have not yet not yet been confirmed, tests suggest (Chapter 14) that these cysts do not parasitize the Solanaceae. The cysts, like those indigenous to Europe, also have to survive extreme climatic conditions as summer temperatures in the Sandveld may rise to above 40°C.

Phylogenetic analyses of the sequence from WK2 clusters this nematode in a group with *Heterodera*, most closely related to *H. bifenestra*, although the morphology of this population is not consistent with that of the *Heterodera* genus. Previous authors have expressed concerns about the placing of *H. bifenestra* in the *Heterodera* group (Ferris, 1998; Subbotin *et al.*, 2001). An in-depth phylogenetic analysis including all the *Heterodera* species, as well as a comparison of the morphology of specimens from WK2 with that of *H. bifenestra* is necessary to resolve this ambiguity, but is not included as it is not within the scope of this study.

Literature

BALDWIN, J.G. (1992). Evolution of cyst and noncyst-forming Heteroderidae. *Annual Review of Phytopathology* 30, 271-290.

BOSSIS, M. & MUGNIÉRY, D. (1993). Specific status of six *Globodera* parasites of solanaceous plants studied by means of two-dimensional gel electrophoresis with a comparison of gel patterns by a computerised system. *Fundamental and Applied Nematology* 16, 47-56.

EDGAR, R.C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research* 32, 1792-1797.

FELSENSTEIN, J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* 39, 783-791.

FERRIS, V.R. (1998). Evolution, phylogeny and systematics. In Sharma, S.B. (Ed.). *The Cyst Nematodes*, Dordrecht, Kluwer Academic, pp. 57–82.

FERRIS, V.R. & FERRIS, J.M. (1992). Integration of classical and molecular approaches in nematode systematics, In: Gommers, F.J. & Maas, P.W.T. (eds.) *Nematology from Molecule to Ecosystem* Dundee, Scotland, European Society of Nematologists Inc., pp. 92-100.

FERRIS, V.R., FERRIS, J.M. & FAGHIHI, J. (1991). Biological diversity from the perspective of molecular biology, In: Dudley, D.C. (ed.). *The Unity of Evolutionary Biology* , Portland,OR, Dioscorides Press, pp. 71-76.

FERRIS, V.R., KRALL, E., FAGHIHI, J. & FERRIS, J.M. (1999) Phylogenetic relationships of *Globodera millefolii*, *G. artemisiae*, and *Cactodera salina* based on ITS region of ribosomal DNA. *Journal of Nematology* 31, 498-507.

FERRIS, V.R., MILLER, L.I., FAGHIHI, J. & FERRIS, J.M. (1995). Ribosomal DNA comparisons of *Globodera* from two continents. *Journal of Nematology* 27, 273-283.

FERRIS, V.R., SABO, A., BALDWIN, J.G., MUNDO-OCAMPO, M., INSERRA, R.N. & SHARMA, S. (2004). Phylogenetic Relationships Among Selected Heteroderoidea Based on 18S and ITS Ribosomal DNA. *Journal of Nematology* 36(3), 202–206.

GRENIER, E., FOURNET, S., PETIT, E. & ANTHOINE, G. (2010). A cyst nematode 'species factory' called the Andes. *Nematology* 12, 163-169.

HANDOO, Z.A., CARTA, L.K., SKANTAR, A.M. & CHITWOOD, D.J. (2012). Description of *Globodera ellingtonae* n. sp. (Nematoda: Heteroderidae) from Oregon. *Journal of Nematology* 44, 40–57.

HYMAN, B.C. & POWERS, T.O. (1991). Integration of molecular data with systematics of plant-parasitic nematodes. *Annual Review of Phytopathology* 29, 89-107.

KATOH, K., KUMA, K., TOH, H. & MIYATA, T. (2005). "MAFFT version 5: improvement in accuracy of multiple sequence alignment". *Nucleic Acids Research* 33, 511-518.

MADANI, M., SUBBOTIN, S.A., WARD, L.J., LI, X. & DE BOER, S.H. (2010). Molecular characterization of Canadian populations of potato cyst nematodes, *Globodera rostochiensis* and *G. pallida* using ribosomal nuclear RNA and cytochrome b genes. *Canadian Journal of Plant Pathology* 32, 252–263.

MADANI, M., KYNDT, T., COLPAERT, N., SUBBOTIN, S.A., GHEYSEN, G. & MOENS, M. (2007). Polymorphism among sugar beet cyst nematode *Heterodera schachtii* populations as inferred from AFLP and ITS-rRNA gene analyses. *Russian Journal of Nematology* 15, 117-128.

MANDURIC, S. & ANDERSSON, S. (2004). The identity of a Swedish *Globodera* (Nematoda: Heteroderidae) population, following comparisons with known populations of *G.artemisiae* (Eroshenko & Kazachenko, 1972) Behrens, 1975. *Russian Journal of Nematology* 12, 39-44.

NEI, M. & KUMAR, S. (2000). Molecular Evolution and Phylogenetics. Oxford University Press, New York..

PICARD, D., SEMPERE, T. & PLANTARD, O. (2008). Direction and timing of uplift propagation in the Peruvian Andes deduced from molecular phylogenetics of highland biotaxa. *Earth and Planetary Science Letters* 271, 326–336.

POWERS, T.O. (2004). Nematode molecular diagnostics: From bands to barcodes. *Annual Review of Phytopathology* 42, 367–83.

POWERS, T.O. & ADAMS, B. (1993). Nucleotide sequences in nematode systematics. In: Lamberti, F. de Giorgi, C & Bird, D. McK. (Eds.). *Advances in Molecular Plant Nematology* , London, UK, Plenum, pp. 9-118.

SABO, A., VOVLAS, N. & FERRIS, V..R. (2001). Phylogenetic relationships based on ribosomal DNA data for four species of cyst nematodes from Italy and one from Syria. *Journal of Nematology* 33, 183–190.

SABO, A, REIS, L.G.L., KRALL, E., MUNDO-OCAMPO, M., & FERRIS, V.R. (2002). Phylogenetic relationships of a distinct species of *Globodera* from Portugal and Two *Punctodera* species. *Journal of Nematology* 34, 263–266.

SAITOU, N. & NEI, M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* 4, 406-425.

SIRCA, S. & UREK, G. (2004). Morphometrical and ribosomal DNA sequence analysis of *Globodera rostochiensis* and *Globodera achilleae* from Slovenia. *Russian Journal of Nematology* 12, 161-168.

SKANTAR, A.M., HANDOO, Z.A., CARTA, L.K. & CHITWOOD, D.J. (2007). Morphological and molecular identification of *Globodera pallida* associated with potato in Idaho. *Journal of Nematology* 39, 133-144.

STONE, A.R. (1979). Co-evolution of nematodes and plants. *Symbolae Botanicae Uppsala* 22, 46-61.

STONE, A.R. (1983). Three approaches to the status of a species complex with a revision of some *Globodera* (Nematoda: Heteroderidae), in Stone, A,R,, Platt, H.M. & Khalil, L.F. *Concepts in Nematode Systematics*, Systematics Association Special Volume No 22. London, UK, Academic Press, pp. 221-33.

STURHAN, D. (2002). Notes on the genus *Cactodera* Krall & Krall, 1978 and proposal of *Betulodera betulae* gen. nov.,comb. nov. (Nematoda: Heteroderidae). *Nematology* 4, 875-882.

STURHAN, D., WOUTS, W.M. & SUBBOTIN, S.A. (2007) An unusual cyst nematode from New Zealand, *Paradolichodera tenuissima* gen. n., sp. n. (Tylenchida: Heteroderidae). *Nematology*, 9, 561-571.

SUBBOTIN, S.A., CID DEL PRADO VERA, I., MUNDO-OCAMPO, M. & BALDWIN, J.G. (2011). Identification, phylogeny and phylogeography of circumfenestrate cyst nematodes (Nematoda: Heteroderidae) as inferred from analysis of ITS-rDNA. *Nematology*. 13(7), 805-824.

SUBBOTIN, S.A, HALFORD, P.D., WARRY, A & PERRY, R.N. (2000). Variations in ribosomal DNA sequences and phylogeny of *Globodera* parasitising solanaceous plants. *Nematology* 2, 591-604.

SUBBOTIN, S.A., VIERSTRAETE, A., DE LEY, P., ROWE, J, WAEYENBERGE, L., MOENS, M. & VANFLETEREN, J.R. (2001). Phylogenetic relationships within the cyst-forming nematodes (Nematoda, Heteroderidae) based on analysis of sequences from the ITS regions of ribosomal DNA. *Molecular Phylogenetics and Evolution* 21, 1–16.

TAMURA, K. & NEI, M. (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution* 10, 512-526.

TAMURA, K., NEI, M., & KUMAR, S. (2004). Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proceedings of the National Academy of Sciences (USA)* 101, 11030-11035.

TAMURA, K., PETERSON, D., PETERSON, N., STECHER, G., NEI, M. & KUMAR, S. (2011). MEGA5: Molecular evolutionary genetics analysis using maximum likelihood,

evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution* 28, 2731-2739.

TANHA MAAFI, Z., SUBBOTIN, S.A. & MOENS, M. (2003). Molecular identification of cyst-forming nematodes (Heteroderidae) from Iran and a phylogeny based on ITS-rDNA sequences. *Nematology* 5, 99-111.

THIERY, M. & MUGNIERY, D. (1996). Interspecific rDNA restriction fragment length polymorphism in *Globodera* species, parasites of solanaceous plants. *Fundamental and Applied Nematology* 19, 471-479.

THOMPSON, J.D., GIBSON, T.J., PLEWNIAK, F., JEANMOUGIN, F. & HIGGINS, D.G. (1997). The CLUSTALX windows interface; flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* 25, 4876-4882.

UEHARA, T., KUSHIDA, A., ITOU, K., NARABU,T. & MOMOTA, Y. (2005). Discrimination of three cyst-forming nematodes of the genus *Globodera* (Nematode: Heteroderidae) from Japan based on PCR-RFLP of ribosomal DNA. *Applied Entomology and Zoology (Japan)* 40, 537-543.

VRAIN, T.C., WAKARCHUCK, D.A., LÈVESQUE, A.C. & HAMILTON, R.I. (1992). Intraspecific rDNA restriction fragment length polymorphism in the *Xiphinema americanum* group. *Fundamental and Applied Nematology* 15, 563-573.

YAN, G. & SMILEY, R.W. (2009). Distinguishing *Heterodera filipjevi* and *H. avenae* using polymerase chain reaction-restriction fragment length polymorphism and cyst morphology. *Phytopathology* 100, 216-224.

Table 4.1. Isolates of indigenous cyst nematodes used in this study.

Nr.	Associated plant genera	Cyst shape	Genbank accession nr.
SP25	*Conicosia, Oncosiphon*	Spheroid, lacking a terminal cone	JQ595409
SK18	*Solanum, Chrysanthemoides*	Spheroid, lacking a terminal cone	KC148543
WK01	*Indogifera, Wiborgia, Lampranthus*	Spheroid, lacking a terminal cone	KF809767
WK02	*Lycium, Solanum, Asparagus*	Spheroid to ovoid with an obtuse terminal cone	KF809768

Table 4.2. Sequence lengths and frequency of nucleotide distribution of ITS regions of selected *Globodera* species and indigenous cyst nematode populations.

Species	ITS1 (bp)	5.8S (bp)	ITS2 (bp)	A (%)	C (%)	G (%)	T (%)
SP25 (JQ595409)	543	171	203	0.204	0.231	0.273	0.292
SK18 (KC148543)	542	171	207	0.205	0.228	0.272	0.295
WK1 (KF809767)	538	171	204	0.216	0.228	0.273	0.284
WK2 (KF809768)	567	171	197	0.201	0.228	0.270	0.302
G. artemisiae (AY519127)	534	171	205	0.208	0.230	0.280	0.281
G. ellingtonae (JF739926)	530	171	199	0.206	0.244	0.276	0.274
G. mexicana (EU006709)	533	172	200	0.209	0.238	0.272	0.282
G. millefolii (HQ260407)	536	171	205	0.211	0.229	0.277	0.283
G. pallida (GU084819)	531	171	199	0.209	0.243	0.273	0.275
G. rostochiensis (FJ212164)	529	171	199	0.207	0.246	0.274	0.274
G. sp. Chile (GU084808)	530	171	199	0.207	0.244	0.273	0.276
G. sp. Portugal (AY090883)	534	170	204	0.216	0.225	0.272	0.287
G. tabacum tabacum (EF153842)	531	171	200	0.206	0.244	0.274	0.276
G. zelandica (HQ260411)	550	173	210	0.207	0.234	0.279	0.281

Table 4.3. Pairwise distances between ITS regions of selected *Globodera* species and indigenous cyst nematode populations. The number of base substitutions per site from between sequences according to the Tamura-Nei model is shown below the diagonal. The number of base differences per sequence from between sequences is shown above the diagonal.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	*G. artemisiae* (AY519127)	-	9	40	34	41	63	51	73	71	66	69	63	62	242
2	*G. millefolii* (HQ260407)	0.010	-	39	33	40	60	50	73	71	66	67	61	62	244
3	WK1 (KF809767)	0.046	0.045	-	48	42	71	53	75	75	71	77	71	69	254
4	SK18 (KC148543)	0.038	0.037	0.055	-	39	67	56	79	77	72	74	69	68	251
5	*G.* sp. Portugal (AY090883)	0.047	0.046	0.048	0.045	-	66	62	85	83	76	78	71	72	251
6	SP25 (JQ595409)	0.074	0.070	0.084	0.079	0.078	-	67	70	70	67	69	64	62	246
7	*G. zelandica* (HQ260411)	0.059	0.058	0.061	0.065	0.073	0.078	-	71	71	67	75	70	63	243
8	*G.* sp. Chile (GU084808)	0.087	0.087	0.089	0.095	0.103	0.083	0.084	-	14	24	33	29	25	237
9	*G. ellingtonae* (JF739926)	0.084	0.084	0.089	0.092	0.100	0.083	0.084	0.016	-	22	31	27	24	231
10	*G. tabacum* (EF153842)	0.078	0.078	0.084	0.085	0.091	0.079	0.079	0.027	0.025	-	30	28	15	233
11	*G. mexicana* (EU006709)	0.082	0.079	0.092	0.088	0.093	0.082	0.089	0.038	0.035	0.034	-	14	33	236
12	*G. pallida* (GU084819)	0.074	0.072	0.084	0.082	0.084	0.076	0.083	0.033	0.031	0.032	0.016	-	29	232
13	*G. rostochiensis* (FJ212164)	0.073	0.073	0.082	0.081	0.086	0.073	0.074	0.029	0.027	0.017	0.038	0.033	-	230
14	WK2 (KF809768)	0.349	0.352	0.371	0.364	0.366	0.357	0.351	0.341	0.330	0.333	0.338	0.331	0.328	-

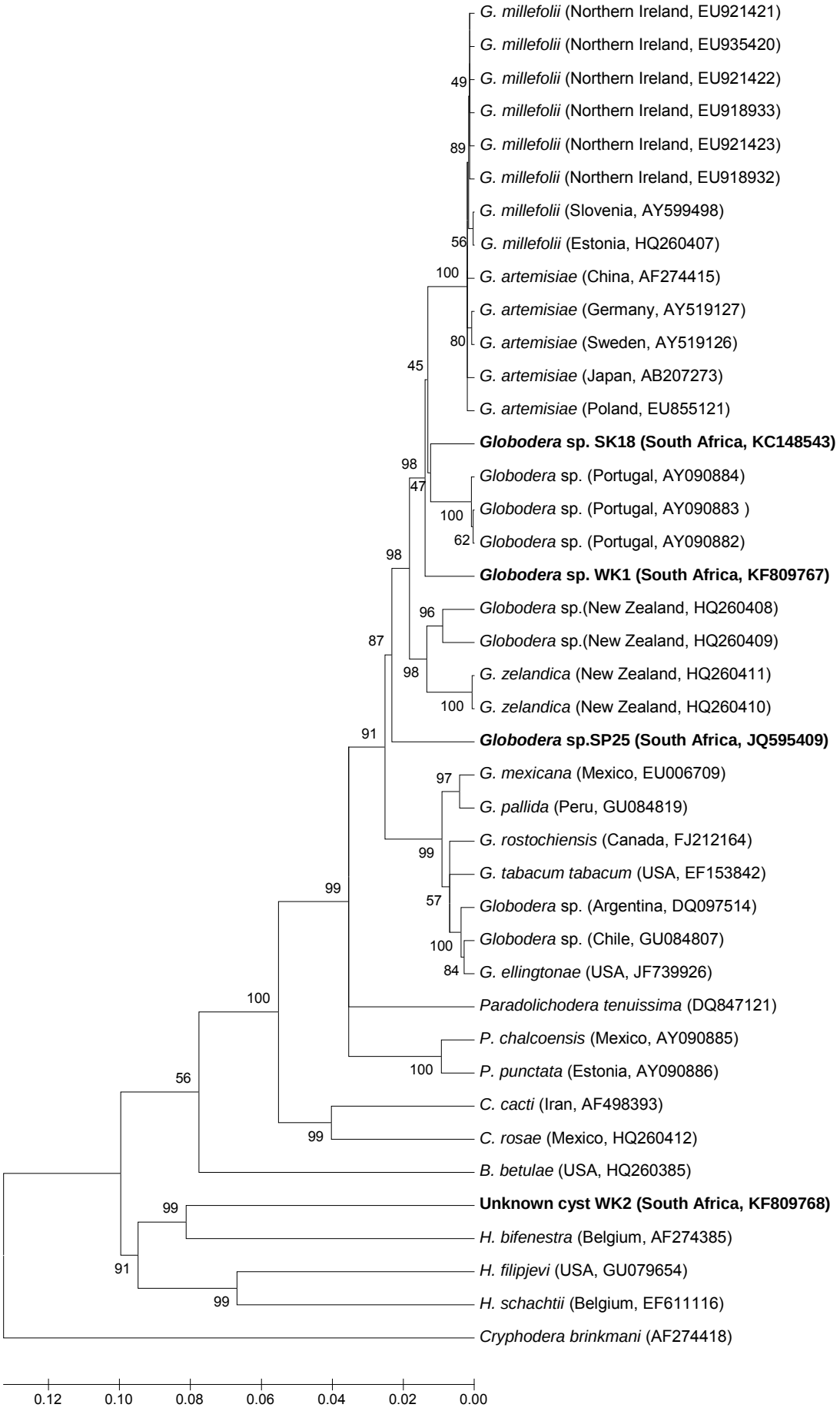

Fig. 4.1. Phylogenetic relationships of cyst-forming nematodes as inferred from ITS-rRNA sequences by using the Neighbor-Joining method. The optimal tree with the sum of branch length = 1.05775527 is shown. The percentage of replicate trees in which the associated taxa cluster together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

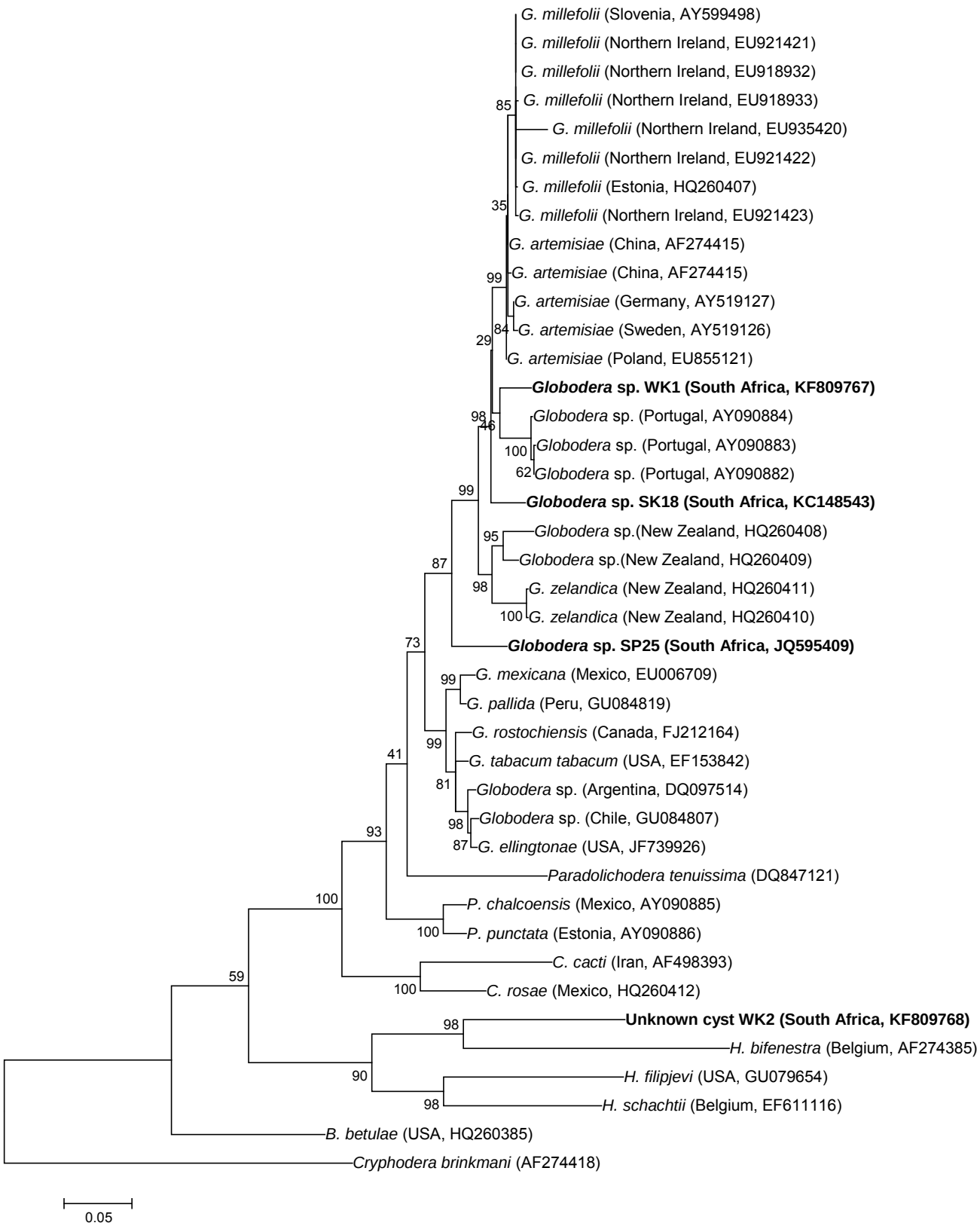

Fig. 4.2. Phylogenetic relationships of cyst-forming nematodes as inferred from ITS-rRNA sequences by using the Maximum Likelihood method. The tree with the highest log likelihood (-9318.7266) is shown. The percentage of trees in which the associated taxa clustere clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

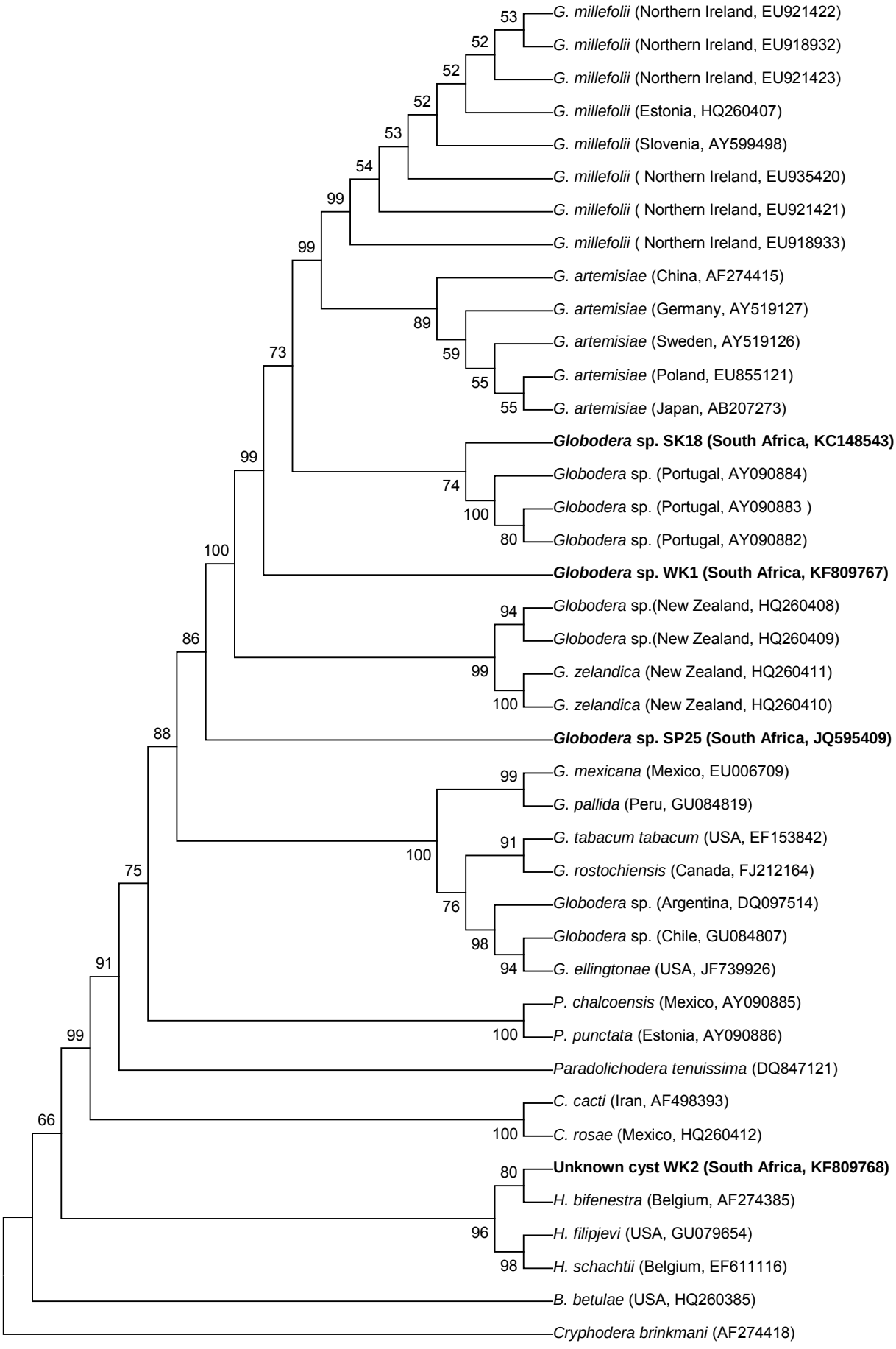

Fig. 4.3. Phylogenetic relationships of cyst-forming nematodes as inferred from ITS-rRNA sequences by using the Maximum Parsimony method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analysed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches.

Chapter 5

Intraspecific variation among South African populations of *Globodera rostochiensis* as revealed by ITS-rDNA and Random Amplified Polymorphic DNA

Introduction

A number of DNA fingerprinting and other molecular techniques have been used to characterise PCN (*Globodera rostochiensis* (Wollenweber, 1923) Skarbilovich, 1959 and *G. pallida* Stone, 1973) populations and to detect isolates with different pathogenicity. Phillips *et al.* (1992) used isozyme analysis and restriction digest of total DNA (RFLP) to determine if natural groups of *G. pallida* could be identified. With one exception, no clear and consistent patterns in terms of virulence and or geographic location could be seen. Zaheer *et al.* (1996) also studied potato cyst-nematodes for genetic variation by using isozyme analysis. The calculation of coefficient of similarity on 15 enzymes and construction of a dendrogram for a range of PCN populations revealed low levels of genetic variation among the *G. rostochiensis* populations. Hinch *et al*. (1998) used high performance capillary electrophoresis to differentiate between pathotypes of *G. rostochiensis* and *G. pallida*. Reproducible polypeptide profiles obtained displayed some characteristic peptides for each of the pathotypes of *G. rostochiensis*.

Pathotypes of *G. rostochiensisis* (Ro1 and Ro2/3) were subjected to polymerase chain reaction amplification using one arbitrary primer (Chacón *et al.*, 1994). This technique resulted in relatively simple DNA profiles that included polymorphic markers known as random amplified polymorphic DNA (RAPD), resulting in a race specific profile that allows

these two races to be differentiated. Genetic relationships between populations of *G. rostochiensis* and *G. pallida* were analysed by Folkertsma *et al.* (1994) using RAPD. They found that only nine of 250 amplified DNA fragments were common to both species. *G. rostochiensis* populations with identical pathotype classifications were distinguishable by a number of unique RAPD fragments. Simple sequence repeat (SSR) primers were used by Blok and Phillips (1995) to investigate genetic diversity between a number of *G. rostochiensis* and *G. pallida* populations. Eight such SSR primers were examined of which three gave reproducible results. The data obtained from these clearly separated the two species and allowed discrimination between the populations within each species. Some of the groupings obtained related to the geographical origin of the populations rather than their virulence characteristics. The high copy fragments generated by the digestion of total DNA using four endonucleases were analysed in 33 populations of potato cyst nematodes from the Canary Islands and Europe (Gonzalez *et al.*, 1995). Large differences were found between species, but no appreciable variation was observed within populations of *G. rostochiensis*.

Folkertsma *et al.* (1996) used Amplified Fragment Length Polymorphism PCR (AFLP) to characterise 24 potato cyst nematode populations, revealing 513 amplified DNA fragments in *G. rostochiensis* by screening 12 primer combinations. The estimated similarity among *G. rostochiensis* populations based on the shared presence of 81 polymorphic DNA fragments averaged 0.587 (0.167 - 0.938). A dendrogram based on the variants expressed by the presence or absence of DNA fragments clustered the *G. rostochiensis* populations into three groups which coincide with their pathotype classification as defined by Kort *et al.* (1977). The three groups were distinguished by 3, 7 and 12 unique DNA fragments, respectively. Genetic variation between populations of *G. pallida*, primarily from Britain, but including populations from continental Europe and South America and two *G. rostochiensis*

populations, was examined using RAPD (Blok *et al*, 1997). Fourteen primers were used and 250 amplification products observed (174 specific to *G. pallida* and 43 to *G. rostochiensis*). In this study the two populations of *G. rostochiensis*, both pathotype Rol, were very similar, but the dissimilarity to *G. pallida* was greater than that found in a Dutch study (Folkertsma *et al.*, 1994). Seven populations of *G. rostochiensis* from the UK and single populations from Germany, the Netherlands and Bolivia were analysed using RAPD-PCR by Bendezu *et al.* (1998). With four primers, the genomic similarity among European populations of *G. rostochiensis* was 82% and among UK populations, 89%. A population from the Netherlands (pathotype Ro3) appeared very different from the UK population (Ro1).

A method based in two-dimensional protein gel electrophoresis has been developed in order to improve the analysis of genetic relationships among populations of *Globodera*. It has been used to estimate genetic divergence among nine populations of *G. pallida* (Fullaondo *et al.*, 2001). Grenier *et al.* (2001) analysed Peruvian potato cyst nematode populations to assess both their inter- and intraspecific similarities using 2-DGE and RAPD datasets. The clear distinction between the Peruvian and European populations was confirmed for *G. pallida.* It was concluded that the primary founders of European populations may have an origin other than that of the Peruvian populations used in their study.

Genetic variability of 32 populations of *G. rostochiensis* and three of *G. pallida* from different regions of Portugal were analysed and compared using RAPD with sixteen primers (Da Conceição *et al.*, 2003). Separation of the two species of PCN was achieved with an average similarity of 43%. Two populations of *G. rostochiensis* appear to be distinct from the main group of this species. Distinct clusters were observed within both species, but the clusters could not be related to the geographic proximity of the populations. Random amplified polymorphic DNA (RAPDs) were also used to investigate the intraspecific

variability among 19 geographic isolates of *Globodera tabacum solanacearum* from Virginia and North Carolina (Syracuse *et al.*, 2004).

Manduric & Andersson (2003) compared sixteen Swedish PCN populations (nine *G. rostochiensis* and seven *G. pallida* populations) with 20 other European populations (nine *G. rostochiensis* and 11 *G. pallida* populations) from Germany, the Netherlands, Norway and the United Kingdom in an AFLP study. Seven AFLP primer combinations were used to amplify genomic DNAs extracted from cysts. A dendrogram based on UPGMA (Unweighted Pair Group Method with Arithmetic Mean) and evaluated by bootstrap analysis, contained two main clusters, one for each species. The Swedish Ro1 populations were very similar to corresponding populations from other parts of Europe. Remaining *G. rostochiensis* populations appeared as a genetically heterogeneous group with two Swedish populations being most dissimilar.

Picard *et al.* (2007) genotyped individuals from 42 Peruvian populations of *G. pallida* using mitochondrial and nuclear molecular markers to investigate its phylogeography. A clear south-to-north phylogeographical pattern was revealed with five well-supported clades. Picard *et al.* (2008) used a molecular phylogeographic and phylochronologic analysis of *G. pallida* to assess the development and timing of regional surface uplift in the Andes. Plantard *et al.* (2008) identified the origins of Western European populations of *G. pallida* with great accuracy using mitochondrial sequences and microsatellite loci.

Hlaoua *et al.* (2008) used RAPD to compare Tunisian and European PCN populations to reveal a possible phylogeny between them and determine the origin of infestations. The RAPD revealed that Tunisian *G. pallida* populations correspond to Pa2/3 pathotypes and

biological results proved that the *G. rostochiensis* populations belong to the Ro1/4 pathotype. Results from a bootstrap analysis showed a high similarity with European PCN populations.

Chrisanfova *et al.* (2008) used polymorphism of the rDNA first intergenic spacer ITS1, to identify populations in Russia as *G. rostochiensis*. Small variations of the ITS1 sequence in different geographic populations of nematodes from different regions of the species world range did not allow isolation of separate groups within the species. Genetic variability of *G. rostochiensis* from three Russian populations was also investigated using RAPD markers (Chrisanfova *et al.*, 2008). It was possible to distinguish a Karelian population from the group consisting of the populations from two adjacent regions. A dendrogram of genetic differences was constructed, and the indices of genetic divergence and partition, as well as the gene flow indices between the nematode samples examined, were calculated. The dendrogram structure, genetic diversity indices, and variations of genetic distances between single individuals in each population from Karelia and Central Russia pointed to genetic isolation and higher genetic diversity of the nematodes from Karelia. Pylypenko *et al.* (2008) compared the mitochondrial Cytochrome B gene from the Ukrainian populations of *G. pallida* with other European and South American populations. The data indicated that the Ukrainian populations were similar to other European populations.

ITS sequence analyses of isolates of PCN in Victoria, Australia have revealed the presence of genetically diverse populations of *G. rostochiensis* in infested fields (Quader *et al.*, 2008). In this study, the sequence similarity amongst isolates of *G. rostochiensis* was high (>99%), indicating once again the common ancestry of the species. However, isolates of Victorian PCN population were different to the European and American genotypes recorded in GenBank which vary little amongst themselves. The pattern of grouping of isolates in the phylogenetic tree from different growers in Victoria indicated the possibility of up to seven

introductions in this region. The phylogenetic analysis inferred by the sequence of the rDNA ITS region confirmed the identity of a cyst nematode from Quebec, Canada as *G. rostochiensis* with 100% match to the *G. rostochiensis* from Russia, Peru, Japan and UK (Yu *et al.*, 2010). This analysis resolved the key monophyletic groups, including *Globodera*, *G. rostochiensis*, *G. pallida* and *G. tabacum.* The ITS regions of the rRNA gene and D2-D3 expansion segments of the 28S rRNA gene were amplified, sequenced and used to characterize several populations of potato cyst nematodes, collected from different areas in Canada (Madani *et al.*, 2010). Sequence analyses of these genes were performed to reveal inter- and intraspecific variation and to compare them with those of potato cyst nematodes from other countries in order to test the possible origin of introduction of PCN into Canada. Although ITS sequence diversity for *G. rostochiensis* populations was only slightly less than that for *G. pallida,* their phylogenetic analysis did not reveal distinct phylogenetic patterns among geographical isolates of *G. rostochiensis,* and relationships between the sequences were unresolved. The relatively low rate of evolution of the ITS-rRNA gene fragment and the mixture ITS haplotypes per individual did not allow them to use it as a reliable genetic marker for the study of recent introduction events. Their analysis did not allow them to determine the origin of Canadian *G. rostochiensis* populations.

Grenier *et al.* (2010) argued that the uplift of the Andes Mountains has triggered a variety of adaptive biotic radiations for Solanaceous plant-parasitic nematodes and has represented a key factor for the evolution and specialisation of *Globodera* species. The variety of related and unrelated hosts amongst *Globodera* species suggests that speciation occurs in the genus by opportunistic parasitising of new host groups.

In this study, the genetic variation between geographical populations of *G. rostochiensis*, using ITS-rDNA and Random Amplified Polymorphic DNA (RAPD), will be investigated.

Materials and Methods

SOURCES OF ISOLATES

Globodera rostochiensis in South Africa is represented by five geographically separate populations. They originate from unique locations in the Sandveld, Ceres, South-Western Cape, Eastern Cape and Gauteng potato-producing areas (Table 5.1). For ease of reference they will henceforth be called the Sandveld, Ceres, Philippi, Hankey and Gauteng populations.

PREPARATION OF DNA TEMPLATES

Cysts were cut open and the juveniles removed. Individual juveniles were handpicked and placed in a 10 µl drop of 1× PCR reaction buffer (16mM $[NH_4]_2SO_4$, 67 mM Tris-HCl pH 8.8, 0.1% Tween-20) containing 60 µg/ml Proteinase K in a sterile PCR tube. The nematode was then cut into small pieces with a sterile scalpel blade. The tube was then incubated at 60°C for 15 minutes, and a further 5 minutes at 95°C. The lysate was stored at -80°C until further use.

AMPLIFICATION OF ITS-RDNA

Two PCR amplification primers that amplify the ITS regions, including the 5.8S ribosomal gene, as well as short parts of the 18S and 28S ribosomal genes were used. The 18S rDNA1

primer (5'-TTGATTACGTCCCTGCCCTTT-3') and 28S rDNA2 primer (5'-TTTCACTCGCCGTTACTAAGG-3') have been described by Vrain *et al*. (1992) for amplification of the ITS regions. The primers were synthesized by Integrated DNA Technologies Inc. (Coralville, Iowa, USA). PCR amplifications were carried out using 5 µl of nematode lysate together with 0.5 µM of each primer, dATP, dCTP, dGTP and dTTP each at 200 µM final concentration, 1 × Taq reaction buffer, 1.5 mM $MgCl_2$ and 1 U Taq polymerase. The final reaction volume was 25 µl. The cycling conditions were as follows: denaturation at 94°C for 20 s, annealing at 55°C for 30 s and extension at 72°C for 45 s, repeated for 30 cycles. A 2 min incubation period at 72°C followed the last cycle in order to complete any partially synthesised strands.

RAPD-PCR

RAPD-PCR was performed using nine decamer primers (Welsh & McClelland, 1990), listed in Table 5.2. The primers were synthesized by Integrated DNA Technologies Inc. (Coralville, Iowa, USA). PCR amplifications were carried out using 1 µl of nematode lysate together with 1.0 µM of primer, dATP, dCTP, dGTP and dTTP each at 200 µM final concentration, 1 × Taq reaction buffer, 1.5 mM $MgCl_2$ and 1 U Taq polymerase. The final reaction volume was 25 µl. The cycling conditions were as follows: denaturation at 94°C for 1 min, annealing at 38°C for 2 min and extension at 72°C for 3 min, repeated for 45 cycles. A 5 min denaturing period preceded the first cycle and a 5 min incubation period at 72°C followed the last cycle in order to complete any partially synthesised strands.

Ten microliter of the amplification products was loaded on a 2% Metaphor® (FMC Bioproducts) agarose gel and separated by electrophoresis. A 100 bp DNA ladder (Fermentas life sciences) was used as a size marker. The DNA banding patterns, stained with SYBR® Gold (Life technologies), were visualised and photographed under UV transillumination. Three independent reactions of each amplication were carried out for each nematode population/primer combination to ensure the reproducibility of the technique.

DNA SEQUENCING

PCR products of the ITS-rDNA regions were cleaned up and sequenced by Inqaba Biotechnical Industries (Pty) Ltd, using an ABI 3500xL Genetic Analyzer with 24 capillaries. A BigDye version 3.1 dye terminator cycle sequencing kit from Applied Biosystems was used for the reactions. Two internal primers, PunQF1 (5'-GAACTGCAGAAACCTTGA -3') and PunQR1 (5'-TTCACCTACAGCTACCTT -3') were designed and used in addition to the PCR primers 18S and 28S to enable the sequencing of the complete PCR products. Sequence assembly and editing was performed on the CLC DNA Workbench 6.7.1 (http://www.clcbio.com).

PHYLOGENETIC ANALYSIS

The ITS-rDNA sequences of the geographic populations of *G. rostochiensis* were aligned with sequences available from Genbank (Ferris *et al*., 1995; Bulman & Marshall, 1997; Szalanski *et al*., 1997; Blok *et al*., 1997; Subbotin *et al.*, 2000; Sirca & Urek, 2004; Uehara *et*

al., 2005; Quader *et al*., 2008; Madani *et al.*, 2010). The sequences (57) were aligned using ClustalX 2.1 with default options (Thompson *et al*., 1997).

Phylogenetic analyses to resolve the relationships between the geographic populations of *G. rostochiensis* were conducted using the Minimum Evolution method (Rzhetsky & Nei, 1992). Evolutionary distances were computed using the Tamura-Nei method (Tamura & Nei, 1993). The ME tree was searched using the Close-Neighbor-Interchange (CNI) algorithm (Nei & Kumar, 2000). The Neighbor-joining algorithm (Saitou & Nei, 1987) was used to generate the initial tree. The confidence intervals for the various branching patterns in the trees was measured with the bootstrap test (BS) with 1000 replicates (Felsenstein, 1985). Evolutionary analyses were conducted in MEGA5 (Tamura *et al.*, 2011).

DATA ANALYSIS OF RAPD BANDING PATTERNS

Molecular weights were calculated and the presence or absence of DNA fragments for each population/primer combination was scored using the GeLAnalyzer (version 2010a) computer program. Differences in band brightness were not taken into account. For each population, a binary data matrix was constructed based on the presence or absence of each DNA fragment.

The resulting matrix was used as input data to the Phylogeny Inference Package (PHYLIP, version 3.5c). Genetic distances (Nei, & Li, 1979) and cluster analysis by the unweighted pair group method using arithmetic averages (UPGMA) were obtained with the RESTDIST and NEIGHBOR programs, respectively. A bootstrap analysis was performed and 1000 bootstrapped data sets and consequent similarity matrices were produced using SEQBOOT and RESTDIST. The NEIGHBOR and CONSENSUS programs in the PHYLIP package were

used to generate the 1000 trees, and then to produce a consensus tree. Dendrograms were constructed with the TREEVIEW program.

Results

AMPLIFICATION OF ITS-RDNA

Amplification of the ITS regions with primers rDNA1 and rDNA2 yielded a single fragment of 1193 bp for *G. rostochiensis.*

PHYLOGENETIC RELATIONSHIPS

Figure 5.1 presents a phylogenetic tree based on the rDNA ITS 1 from a multiple alignment of 57 nucleotide sequences. There were a total of 1211 positions in the final dataset. Using *G. artemisiae* as an out-group taxon, this tree inferred two highly supported monophyletic groups. All 55 populations of *G.rostochiensis* from different regions of the world were in a clade, with 97% bootstrap support.

RAPD ANALYSIS

The amplified DNA fragments were in the range of 200 to 1850 bp, with nine (OPG-2) to 40 (OPG-6) amplification products for each of 9 primers, yielding a total of 166 markers for the five nematode isolates used in the analysis. Figure 5.2 presents the RAPD profiles that resulted from one of the amplifications (OPG-6) performed in order to illustrate the banding

patterns observed. Distinction of some populations of *G. rostochiensis* was possible with some of the primers. As an example, primer OPG-6 yielded patterns with several bands that differentiated the populations from each other. The genetic distances between the *G. rostochiensis* populations, as calculated with the method of Nei and Li (1979) ranged from 0.014 to 0.021.

In the consensus tree, derived from the banding patterns, the Sandveld population clustered apart from the other populations within the species (Fig. 5.3). The other populations clustered together with a bootstrap value of 100%. Within that clade, the Hankey and Gauteng populations clustered together with a bootstrap value of 79%, while the Ceres and Philippi populations clustered together with an even lower bootstrap value of 59%, indicating no clear differentiation between the groupings.

Discussion

The phylogenetic analysis of geographical isolates of *G. rostochiensis* did not reveal distinct phylogenetic patterns among among geographical isolates of this species, and relationships between these *G. rostochiensis* sequences were largely unresolved. Madani *et al.* (2010) and Yu *et al.* (2010) came to the same conclusion in their analyses, citing the fact that *G. rostochiensis* populations contain a mixture of ITS haplotypes, with a different ratio for different populations, and the relatively low rate of evolution of the ITS-rRNA gene fragment as reasons why it is not suitable to use as a reliable genetic marker for the study of recent introduction events. They could not support the conclusion made by Quader *et al.* (2008) that possibly up to seven introductions of *G. rostochiensis* occurred into Australia. The results of

these analyses of the South African populations are similar and also do not allow the determination of the origin of South African *G. rostochiensis* populations.

The use of RAPD PCR for the analysis of variability of five South African populations of *G. rostochiensis* shows their genetic heterogeneity. Using random primers, it was possible to distinguish the populations from each other. Clustering patterns presented in the consensus tree, suggests that the Sandveld population is genetically different from the other populations within the species. The possibility that these genetic differences are indicative of the existence of different pathotypes being present will be explored in Chapter 12. Further information about the origins, genetic diversity and its linkage with geographic location can be deducted from RAPD studies, if more local isolates together with isolates from other continents are studied in the same manner. However, we have to take into consideration that, although RAPD-PCR is reproducible within the same laboratory, it is notorious for its variable results between laboratories and operators.

Literature

BENDEZU, I.F., EVANS, K., BURROWS, P.R., DE POMERAI, D. & CANTO-SAENZ, M. (1998). Inter and intra-specific genomic variability of the potato cyst nematodes *Globodera pallida* and *G. rostochiensis* from Europe and South America using RAPD-PCR. *Nematologica* 44, 49-61.

BLOK, V.C. & PHILLIPS, M.S. (1995). The use of repeat sequence primers for investigating genetic diversity between populations of potato cyst nematodes with differing virulence. *Fundamental and Applied Nematology* 18 (6), 575-582.

BLOK, V.C., PHILLIPS, M.S. & HARROWER, B.E. (1997). Comparison of British populations of potato cyst nematodes with populations from continental Europe and South America using RAPDs. *Genome* 40, 286-293.

BULMAN, S.R. & MARSHALL, J.W. (1997). Differentiation of Australasian potato cyst nematode (PCN) populations using the polymerase chain reaction (PCR). *New Zealand Journal of Crop and Horticultural Science* 25, 123-129.

CHACÓN, M.R., RODRIGUEZ, E., PARKHOUSE, R.M., BURROWS, P.R. & GARATE, T. (1994). The differentiation of parasitic nematodes using random amplified polymorphic DNA. *Journal of Helminthology* 68, 109-13.

CHRISANFOVA, G.G., CHARCHEVNIKOV, D.A., POPOV, I.O., ZINOVIEVA, S.V., & SEMYENOVA, S.K. (2008). Genetic variability and differentiation of three Russian populations of Potato Cyst Nematode *Globodera rostochiensis* as revealed by nuclear markers. *Russian Journal of Genetics* 44, 533–538.

DA CONCEIÇÃO, I.L., SANTOS, M.C.V., DE OLIVEIRA ABRANTES, I.M. & DE ALMEIDA SANTOS, M.S (2003). Using RAPD markers to analyse genetic diversity in Portuguese potato cyst nematode populations. *Nematology* 5, 137-143.

FELSENSTEIN, J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* 39, 783-791.

FERRIS, V.R., MILLER, L.I., FAGHIHI, J. & FERRIS, J.M. (1995). Ribosomal DNA comparisons of *Globodera* from two continents. *Journal of Nematology* 27, 273-283.

FOLKERTSMA, R.T., ROUPPE VAN DER VOORT, J.N., VAN GENT-PELZER, M.P.E., DE GROOT, K.E., VAN DEN BOS, W.J., SCHOTS, A., BAKKER, J. & GOMMERS, F.J. (1994). Inter- and intraspecific variation between populations of *Globodera rostochiensis* and *G. pallida* revealed by random amplified polymorphic DNA. *Phytopathology* 84, 807-811.

FOLKERTSMA, R.T., ROUPPE VAN DER VOORT, J.N., DE GROOT. K.E., VAN ZANDVOORT, P.M., SCHOTS, A., GOMMERS, F.J., HELDER, J. & BAKKER, J. (1996). Gene pool similarities of potato cyst nematode populations assessed by AFLP analysis. *Molecular Plant-Microbe Interactions* 9, 47-54.

FULLAONDO, A., VICARIO, A., AGUIRRE, A., BARRENA, I. & SALAZAR, A. (2001). Quantitative analysis of two-dimensional gel electrophoresis protein patterns: a method for studying genetic relationships among *Globodera pallida* populations. *Heredity* 87, 266-272.

GONZALEZ, J.A., PHILLIPS, M.S. & TRUDGILL, D.L. (1995). RFLP analysis in Canary Islands and North European populations of potato cyst nematodes (*Globodera* spp.). I. Analysis of high copy fragments. *Nematologica* 41, 468-479.

GRENIER, E., BOSSIS, M., FOUVILLE, D., RENAULT, L. & MUGNIÉRY, D. (2001). Molecular approaches to the taxonomic position of Peruvian potato cyst nematodes and gene pool similarities in indigenous and imported populations of *Globodera*. *Heredity* 86, 277-290.

GRENIER, E., FOURNET, S., PETIT, E. & ANTHOINE, G. (2010). A cyst nematode 'species factory' called the Andes. *Nematology* 12, 163-169.

HINCH, J.M., ALBEDI, F., SMITH, S.C., WOODWARD, J.R. & EVANS, K. (1998). Discrimination of European and Australian *Globodera rostochiensis* and *G. pallida* pathotypes by high performance capillary electrophoresis. *Fundamental and Applied Nematology* 21(2), 123-128.

HLAOUA, W., HORRIGUE-RAOUANI, N., FOUVILLE, D. & MUGNIÉRY, D. (2008). Morphological and molecular characterisation of potato cyst nematode populations from Tunisia and survey of their probable geographical origin. *Biotechnology* 7(4), 651-659.

KORT, J., ROSS, H., RUMPENHORST, H.J. & STONE, A.R. (1977). An international scheme for identifying and classifying pathotypes of potato cyst nematodes *Globodera rostochiensis* and *G. pallida*. *Nematologica* 23, 333-339.

MADANI, M., SUBBOTIN, S.A., WARD, L.J., LI, X. & DE BOER, S.H. (2010). Molecular characterization of Canadian populations of potato cyst nematodes, *Globodera rostochiensis* and *G. pallida* using ribosomal nuclear RNA and cytochrome b genes. *Canadian Journal of Plant Pathology* 32, 252–263.

MANDURIC, S. & ANDERSSON, S. (2003). Potato cyst nematodes (*Globodera rostochiensis* and *G. pallida*) from Swedish potato cultivation – an AFLP study of their genetic diversity and relationships to other European populations. *Nematology* 5, 851-858.

NEI, M. & KUMAR, S. (2000). Molecular Evolution and Phylogenetics. Oxford University Press, New York..

NEI, M. & LI, W.H. (1979). Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proceedings of the National Academy of Sciences* 76, 5629 – 5273.

PHILLIPS, M.S., HARROWER, B.E., TRUDGILL, D.L., CATLEY, M.A. & WAUGH, R. (1992). Genetic variation in British populations of *Globodera pallida* as revealed by isozyme and DNA analyses. *Nematologica* 38, 304-319.

PICARD, D., SEMPERE, T. & PLANTARD, O. (2007). A northward colonisation of the Andes by the potato cyst nematode during geological times suggests multiple host-shifts from wild to cultivated potatoes. *Molecular Phylogenetics and Evolution* 42, 308–316.

PICARD, D., SEMPERE, T. & PLANTARD, O. (2008). Direction and timing of uplift propagation in the Peruvian Andes deduced from molecular phylogenetics of highland biotaxa. *Earth and Planetary Science Letters* 271, 326–336.

PLANTARD, O., PICARD, D., VALETTE, S., SCURRAH, M., GRENIER, M. & MUGNIÉRY, D. (2008). Origin and genetic diversity of Western European populations of the potato cyst nematode (*Globodera pallida*) inferred from mitochondrial sequences and microsatellite loci. *Molecular Ecology* 17, 2208–2218.

PYLYPENKO, L., PHILLIPS, M.S. & BLOK, V. (2008). Characterisation of two Ukrainian populations of *Globodera pallida* in terms of their virulence and mtDNA, and the biological assessment of a new resistant cultivar Vales Everest. *Nematology* 10(4), 585-590.

QUADER, M., NAMBIAR, L. & CUNNINGTON, J. (2008). Conventional and real-time PCR-based species identification and diversity of potato cyst nematodes (*Globodera* spp.) from Victoria, Australia. *Nematology* 10, 471-478.

RZHETSKY, A. & NEI, M. (1992). A simple method for estimating and testing minimum evolution trees. *Molecular Biology and Evolution* 9, 945-967.

SAITOU, N. & NEI, M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* 4, 406-425.

SIRCA, S. & UREK, G. (2004). Morphometrical and ribosomal DNA sequence analysis of *Globodera rostochiensis* and *Globodera achilleae* from Slovenia. *Russian Journal of Nematology* 12, 161-168.

SUBBOTIN, S.A, HALFORD, P.D., WARRY, A & PERRY, R.N. (2000). Variations in ribosomal DNA sequences and phylogeny of *Globodera* parasitising solanaceous plants. *Nematology* 2, 591-604.

SYRACUSE, A.J., JOHNSON, C.S., EISENBACK, J.D., NESSLER, C.L. & SMITH, E.P. (2004). Intraspecific variability within *Globodera tabacum solanacearum* using random amplified polymorphic DNA. *Journal of Nematology* 36, 433–439.

SZALANSKI, A.L., SUI, D.D., HARRIS, T.S. & POWERS, T.O. (1997). Identification of Cyst Nematodes of Agronomic and Regulatory Concern with PCR-RFLP of ITS1. *Journal of Nematology* 29, 255-267.

TAMURA, K. & NEI, M. (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution* 10, 512-526.

TAMURA, K., PETERSON, D., PETERSON, N., STECHER, G., NEI, M. & KUMAR, S. (2011). MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution* 28, 2731-2739.

THOMPSON, J.D., GIBSON, T.J., PLEWNIAK, F., JEANMOUGIN, F. & HIGGINS, D.G. (1997). The CLUSTALX windows interface; flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* 25, 4876-4882.

UEHARA,T., KUSHIDA,A., ITOU,K., NARABU,T. & MOMOTA,Y. (2005). Discrimination of three cyst-forming nematodes of the genus *Globodera* (Nematode: Heteroderidae) from Japan based on PCR-RFLP of ribosomal DNA. *Applied Entomology and Zoology (Japan)* 40, 537-543.

VRAIN, T.C., WAKARCHUCK, D.A., LÈVESQUE, A.C. & HAMILTON, R.I. (1992). Intraspecific rDNA restriction fragment length polymorphism in the *Xiphinema americanum* group. *Fundamental and Applied Nematology* 15, 563-573.

WELSH, J. & MCCLELLAND, M. 1990. Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Research* 18, 7213-7218.

YU, Q. , YE, W. , SUN, F. & MILLER, S. (2010). Characterization of *Globodera rostochiensis* (Tylenchida: Heteroderidae) associated with potato in Quebec, Canada. *Canadian Journal of Plant Pathology*, 32(2), 264 — 271.

ZAHEER, K., FLEMING, C. & TURNER, S.J. (1996). Genetic Variation in Potato Cyst Nematodes in Northern Ireland. *Biochemical Systematics and Ecology* 24(6), 509-519.

Table 5.1: South African populations of *Globodera rostochiensis* used in this study.

Name	Farm	Region	GPS
Sandveld	Nuwerus	Sandveld	S 32°19.233'; EO 18°25.025'
Ceres	Alhambra	Ceres	S 33°22.001'; EO 19°32.022'
Philippi	Nooitgedacht	South-Western Cape	S 34°02.086'; EO 18°34.172'
Hankey	Ripple Hill	Eastern Cape	S 33°45.993'; EO 24°48.404'
Gauteng	Chadinha bros	Gauteng	S 26°07.421'; EO 27°34.856'

Table 5.2: Sequences of Decamer RAPD primers used to assess genetic variation of South African populations of *Globodera rostochiensis*.

Primer	Nucleotide sequence
OPG-2	5'- GGCACTGAGG -3'
OPG-5	5'- CTGAGACGGA -3'
OPG-6	5'- GTGCCTAACC -3'
OPG-8	5'- TCACGTCCAC -3'
OPG-9	5'- CTGACGTCAC -3'
OPG-10	5'- AGGGCCGTCT -3'
OPG-12	5'- CAGCTCACGA -3'
OPG-16	5'- AGCGTCCTCC -3'
OPG-19	5'- GTCAGGGCAA -3'

Fig. 5.1. Phylogenetic relationships of *Globodera rostochiensis* populations as inferred from ITS-rRNA sequences by using the Minimum Evolution method. The optimal tree with the sum of branch length = 0.13470808 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Values lower than 50% are not shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

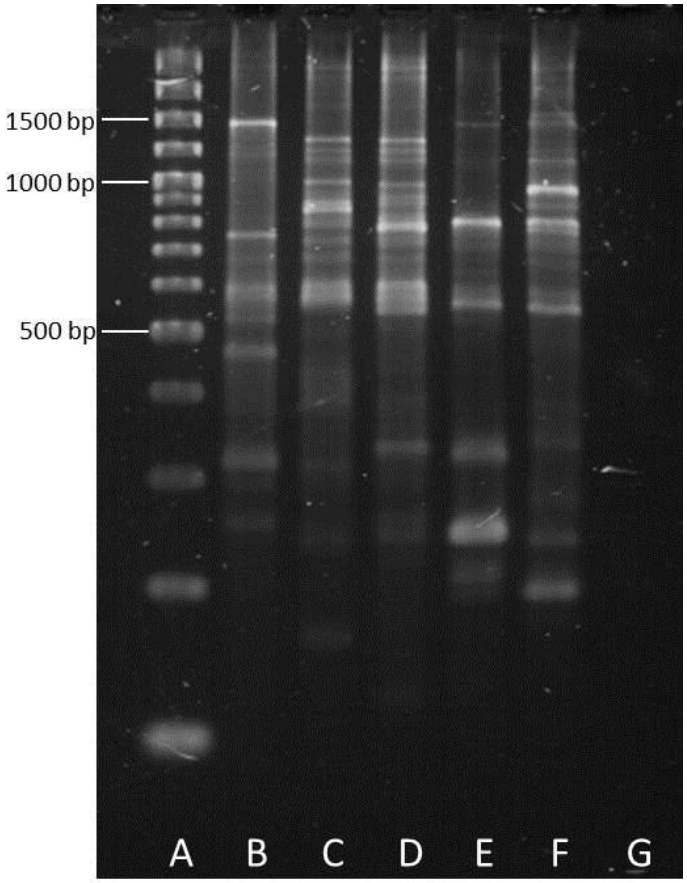

Fig. 5.2 RAPD patterns resulting from amplification with primer OPG-6 from single juveniles of *Globodera rostochiensis*. A: 100 bp DNA marker. B: Sandveld population. C: Ceres population. D: Philippi population. E: Hankey population. F: Gauteng population. G: Blank.

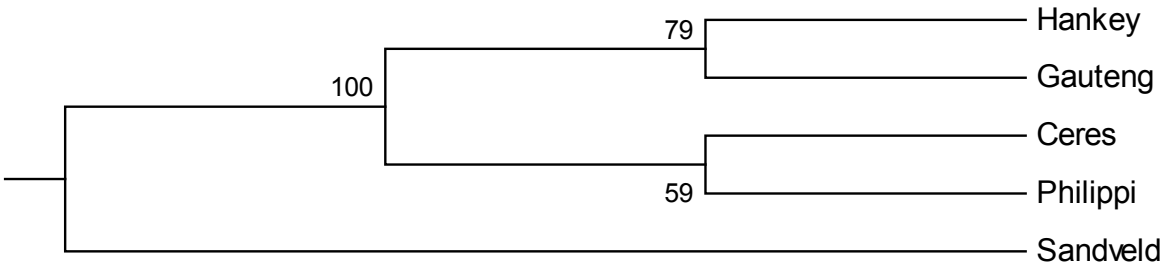

Fig. 5.3. Relationships among 5 *Globodera rostochiensis* populations based on 166 RAPD markers. Consensus tree from 1000 bootstrap trees are shown. Numbers indicate % trees with the particular branching pattern shown. The original dendrogram of similarities was constructed with Nei distances and UPGMA.

Chapter 6

Molecular diagnostic methods for the identification of South African *Globodera* species (Nematoda: Heteroderidae)

Introduction

The presence of new *Globodera* cysts in South Africa (Chapter 2) has complicated the detection of the quarantine nematode, *Globodera rostochiensis* (Wollenweber, 1923) Skarbilovich, 1959 in regulatory samples from these areas. A quick, reliable diagnostic method is needed that would allow growers and regulatory services to discriminate between quarantine and non-quarantine nematodes in samples from these regions.

The morphological identification of nematode species is a time-consuming and skilled process at best. The advantages of a molecular diagnostic system based on the analysis of DNA include speed, sensitivity, accuracy and specificity. The concept of using a targeted "piece" of DNA to identify all life forms on earth, "DNA barcoding", has an essential practical application in Nematology. For these reasons, the development of molecular diagnostic tests has been a priority of research for nematologists in the past.

Different genes and non-coding regions have been identified as possible candidates for use in DNA barcoding for nematodes, including the ITS regions of rDNA (Powers, 2004). PCR amplification combined with restriction enzyme analysis (PCR-RFLP) can provide a powerful diagnostic tool, which is particularly suited to nematode diagnostics, where even DNA from single nematodes can be amplified and analysed further (Powers & Harris, 1993).

The internal transcribed spacers, ITS1 and ITS2, located between the repeating array of nuclear 18S and 28S rRNA genes and separated by the 5.8S rRNA gene, are variable genetic markers, which have been used successfully for the analysis of population structure and systematics in a wide range of organisms. Fleming *et al.* (1993), using primers located in the 28S and 18S genes, designed by Vrain *et al.* (1992), amplified PCN DNA, generating a 1200 bp region encompassing the ITS1 and ITS2 regions and the 5.8S gene. Restriction enzyme digestion of this PCR product revealed the presence of both inter- and intra-specific sequence variation. This approach has been extended by Thiery and Mugniery (1996) to examine the relationships between *G. rostochiensis*, *G. pallida* and the *G. tabacum* complex. Their data demonstrated that these *Globodera* species could be identified by using PCR-RFLP.

The value of rDNA in cyst-nematode diagnostics was also indicated in a comparative study of ribosomal sequence variation from PCN, the *G. tabacum* complex and other *Globodera* species by Ferris *et al*. (1995). Szalanski *et al.* (1997) and Powers *et al.* (1997) evaluated the potential of ITS size and sequence variation as a means of identifying a wide range of free-living, insect- and plant-parasitic nematode groups, including *Globodera, Punctodera, Heterodera* and *Cactodera*. Using two sets of primers, they were able to amplify ITS1 and ITS2 regions and demonstrate that PCR product size could be used to distinguish many nematode genera. Subsequent restriction digestion of the PCR products could then resolve genera exhibiting products of similar size, as well as separating out many specimens at the species level. Other authors to follow this ITS-RFLP approach to distinguish between *Globodera* spp. were Vejl *et al.* (2002); Širca *et al.* (2003); Uehara *et al.* (2005); Knoetze *et al.* (2006) and Skantar *et al.* (2007).

Mulholland *et al*. (1996) described a multiplex PCR-based method, allele-specific amplification, which targeted the ITS1 and 5.8S ribosomal genes and enabled the

identification of *G. pallida, G. rostochiensis* and mixtures of the two species. Unlike standard PCR-based approaches, the technique uses three primers in each PCR reaction, one a universal primer, which anneals to DNA of both species, and two species-specific primers, each designed to mismatch at the 3' end of either *G. pallida* or *G. rostochiensis*. Amplification of *G. pallida* DNA resulted in the production of a 391 bp product, which is distinguished easily using agarose electrophoresis from the 238 bp product generated from *G. rostochiensis* DNA. A mixture of DNA from the two species resulted in the generation of both products. This technique is particularly appropriate for diagnostics, as a sample of PCN should always generate at least one PCR product (i.e. a negative result indicates a failed test) and only a single PCR test is required for each sample, contrasting with traditional PCR, which requires two reactions per diagnosis.

Bulman & Marshall (1997) also reported on the successful use of multiplex PCR in amplifying species-diagnostic rRNA gene sequences for potato cyst nematodes. Using three PCR primers, they found that low levels (1 in 100) of one species in a species mixture could be detected using their procedure. These primers are not necessarily specific for *Globodera* and should only be used on specimens that were morphologically identified as *Globodera.* Pylypenko *et al.* (2005), Skantar *et al*. (2007) and Quader *et al*. (2008) also used the primers of Bulman & Marshall (1997) to distinguish between *Globodera* species. Species-specific primers that can positively identify the tobacco cyst nematode *Globodera tabacum* were also developed by Skantar *et al*. (2007), providing a new assay for distinguishing this species from *G. pallida* and *G. rostochiensis.*

In silico analysis by Subbotin *et al* (2011) of ITS-rDNA from *Globodera* spp. showed that the sequence of the *G. pallida* specific primer, developed by Bulman and Marshall (1997) was well matched with the corresponding sequence region for *G. mexicana.* Thus, false positive

results could be obtained if this primer is used in PCR with a *G. mexicana* sample. The *G. rostochiensis* specific primer developed by Bulman and Marshall (1997) was also well matched with the corresponding sequence regions for the ITS of *G. artemisiae, G. millefolii* and *Globodera* sp. from Portugal, as well as a *Globodera* sp. from South Africa, potentially giving false-positive results with these samples. Also, additional multiplex primers, targeting *G. tabacum*, proposed by Skantar *et al*. (2007) was well matched with the corresponding sequence regions of *G. artemisiae* and *G.millefolii*, and *Globodera* spp. from Portugal and South Africa, once again potentially leading to false-positive results. However, a modification of these primers by Nakhla *et al*., (2010) was specific for *G. tabacum*. Bates *et al.* (2002) used the primers of Bulman & Marshall (1997) to develop a semi-quantitative assay to measure the relative proportions of *G. rostochiensis* and *G. pallida* in a sample. The method depends on a competitive multiplex PCR where the products of each species can be separated by their distinct melting temperatures.

Madani *et al.* (2005) applied the primers of Bulman & Marshall (1997) for quantitative detection of *G. pallida* using real-time PCR. Real-time or quantitative PCR enables both detection and quantification of specific sequences in the reaction. The measurement is made after each amplification cycle, and this is the reason why this method is called real time PCR. Quantitative PCR is carried out in a thermal cycler with the capacity to illuminate each sample with a beam of light of a specified wavelength and detect the fluorescence emitted by an excited fluorochrome. Quader *et al.* (2008) also used the primers of Bulman & Marshall (1997) in a real time PCR. Despite variation in melting peaks, the real-time PCR products provided consistent diagnostic bands for *G. rostochiensis*, thereby indicating the limitation of melting curve analysis for more than 200 bp PCR products. The real-time PCR was able to detect eight times lower levels of DNA than conventional PCR.

Later, Madani *et al.* (2008) developed another multiplex real-time PCR test, based on ITS sequences, with primers and probes modified with locked nucleic acids, which proved to be superior for identification of one or more *Globodera* spp. in samples containing DNA from cysts and second-stage juveniles. The test was specific for *G. rostochiensis, G. pallida* and *G. tabacum tabacum.* Toyota *et al.* (2008) designed primers for the quantitative detection of *G. rostochiensis* using real-time PCR, but their primers were not specific to *G. rostochiensis* and would also amplify DNA from other cyst nematodes. Nowaczyk *et al.* (2008) designed species-specific primers for the differentiation of *G. rostochiensis* and *G. artemisiae* based upon real-time polymerase chain reaction with the use of TaqMan probes, which proved to be sensitive and species-specific. TaqMan primer-probe sets were also developed for the detection and identification of *G. pallida* and *G. rostochiensis* by Nakhla *et al.* (2010), using a two-tube, multiplex real-time PCR. One tube contained a primer-probe set specific for *G. pallida,* multiplexed with another primer-probe set specific for *G. rostochiensis*. A second tube consisted of the *G. pallida*-specific primer-probe set multiplexed with a primer-probe set specific for *G. tabacum.*

The development of DNA-based diagnostic methods for *Globodera* species was not limited to amplification of the ITS regions. Shields *et al.* (1996) used the polymerase chain reaction to amplify a region between the 5S rRNA and spliced leader RNA genes in *G. rostochiensis* and *G. pallida*. *G. rostochiensis* and *G. pallida* were identified by Clapp *et al*. (2000) using the second internal transcribed spacer (ITS2) sequence variation detected by polymerase chain reaction-single-strand conformational polymorphism (PCR-SSCP). The ITS2 region was sufficiently variable within the taxa investigated to allow species to be separated on the basis of minor sequence variation. However, pathotypes of *G. rostochiensis* and *G. pallida* could not be distinguished. RAPD fragments specific for *G. rostochiensis* and *G. pallida*

were identified by Fullaondo *et al.* (1999). These were used to derive two primer combinations for PCR which amplify only one specific band in each species. The primer combinations were evaluated on 39 samples of PCN populations containing all currently described pathotypes as well as some South American PCN populations. This method for species identification was highly sensitive and allowed the detection of species mixtures in a sample. The heat-shock gene, Hsp90, was targeted as a new variable genomic region for identification and discrimination of *G. pallida, G. rostochiensis and G.tabacum tabacum* by Madani *et al.* (2011). General and specific primers and probes for each species were derived from the consensus and nonconsensus regions of the aligned sequences, respectively. A triplex conventional PCR assay, using a general forward and reverse or three specific reverse primers, as well as a real-time PCR using general primers and specific TaqMan probes, were developed. Results confirmed the usefulness of Hsp90 as a new additional gene target and showed that several different test options could be used for discrimination of PCN. Yu *et al.* (2011) cloned a new chorismate mutase gene from *Globodera tabacum* and further characterized the gene structure in both *G. tabacum* and *G. pallida*. Based on genomic sequence variations observed, they developed TaqMan real-time PCR assays that provided a highly specific and sensitive identification of each *Globodera* species, revealing a new application of using the chorismate mutase gene as a valuable diagnostic marker for plant-parasitic nematodes.

In this study, the possibility of utilising the ITS regions of rDNA to develop a quick and reliable PCR-based diagnostic test for the discrimination of *Globodera* spp. in South Africa was investigated.

Materials and Methods

NEMATODE POPULATIONS

The sequences of *G. rostochiensis* used in this chapter originate from the Sandveld population (see Chapter 4). Since *G. pallida* is not known to occur in South Africa, DNA was obtained from L. Waeyenberge from the Agricultural Research Centre, Department of Crop Protection, Merelbeke, Belgium. Isolates from indigenous populations of cysts were collected in the Cape Floristic Kingdom of South Africa (See Chapter 2). The sequences from indigenous *Globodera* species used in this chapter are the same as in Chapter 4. For ease of reference the isolates will be referred to as SP25 (*G. capensis)*, SK18 and WK1.

PREPARATION OF DNA TEMPLATES

Cysts were cut open and the juveniles removed. Individual juveniles were handpicked and placed in a 10 µl drop of 1× PCR reaction buffer (16mM $[NH_4]_2SO_4$, 67 mM Tris-HCl pH 8.8, 0.1% Tween-20) containing 60 µg/ml Proteinase K in a sterile PCR tube. The nematode was then cut into small pieces with a sterile scalpel blade. The tube was then incubated at 60°C for 15 min, and a further 5 min at 95°C. The lysate was stored at -80°C until further use.

POLYMERASE CHAIN REACTION

Two PCR amplification primers that amplify the ITS regions, including the 5.8S ribosomal gene, as well as short parts of the 18S and 28S ribosomal genes were used. The rDNA1 primer and rDNA2 primer has been described by Vrain *et al*. (1992) for amplification of the ITS regions. The ITS1 region alone, including short parts of the 18S and 5.8S ribosomal genes, were also amplified by using the rDNA1 primer and the rDNA1.58 primer that was designed by comparative sequence alignments of various nematode species by Szalanski *et al*. (1997). The primers used in this study are listed in Table 6.1. The primers were synthesized by Integrated DNA Technologies Inc. (Coralville, Iowa, USA).

PCR amplifications were carried out with 5 µl of nematode lysate together with 0.5 µM of each primer, dATP, dCTP, dGTP and dTTP each at 200 µM final concentration, 1 × Taq reaction buffer, 1.5 mM $MgCl_2$ and 1 U Taq polymerase. The final reaction volume was 25 µl. The cycling conditions were as follows: denaturation at 94°C for 20 s, annealing at 55°C for 30 s and extension at 72°C for 45 s, repeated for 30 cycles. A 2 min incubation period at 72°C followed the last cycle in order to complete any partially synthesised strands.

DNA SEQUENCING

PCR products of the ITS-rDNA regions were cleaned up and sequenced by Inqaba Biotechnical Industries (Pty) Ltd, using an ABI 3500xL Genetic Analyzer with 24 capillaries. A BigDye version 3.1 dye terminator cycle sequencing kit from Applied Biosystems was used for the reactions. Two internal primers, PunQF1 and PunQR1 were designed and used in addition to the PCR primers 18S and 28S to enable the sequencing of the complete PCR

products. Sequence assembly and editing was performed on the CLC DNA Workbench 6.7.1 (http://www.clcbio.com).

SEQUENCE ALIGNMENT AND PRIMER DESIGN

The ITS-rDNA sequences of *G. rostochiensis*, SP25, SK18 and WK1 were aligned with the CLC DNA Workbench (http://www.clcbio.com). Primers sagU1, sagR1 and sagP1 were optimised by extension of the primers designed by Vrain *et al.* (1992) and Bulman & Marshall (1997) in order to facilitate melting temperatures that were compatible in a multiplex PCR. Primers sagS1 and sagR2 were newly designed after scrutinisation of the ITS1 region of different *Globodera* spp. (Fig. 6.1).

RESTRICTION FRAGMENT LENGTH POLYMORPHISMS

Almost 1500 restriction enzymes were screened for their ability to discriminate between South African *Globodera* species by virtual digestion of the ITS1 region as amplified by primers 18S and 5.8S using the CLC DNA Workbench 6.7.1 (http://www.clcbio.com). The enzymes were evaluated for their ability to produce species-specific fragments, but enzymes that have too many restriction sites, no restriction sites for one or more of the species or have restriction sites too closely situated, were discarded.

To verify, the results from *in silico* digestion, the amplified ITS1 regions were digested with *Rsa* I. Ten µl of each of the PCR products were digested with 10 U of the restriction enzyme in the appropriate buffer, according to the manufacturer's instructions, in a total volume of 20

µl. The digested DNA was loaded on a 2% agarose gel, separated by electrophoresis, and visualised by ethidium bromide staining.

Results

Amplification of the ITS regions

Amplification of the ITS regions, including the 5.8S ribosomal gene, as well as short parts of the 18S and 28S ribosomal genes with primers rDNA1 and rDNA2 yielded a single fragment of 1193 bp, 1210 bp, 1213 bp and 1205 bp for *G. rostochiensis,* SP25, SK18 and WK1, respectively. Amplification of the The ITS1 region alone, including short parts of the 18S and 5.8S ribosomal genes with primers rDNA1 primer and rDNA1.58 yielded a single fragment of 754 bp, 767 bp, 766 bp and 761 bp for *G. rostochiensis*, SP25, SK18 and WK1, respectively. Figure 6.2 shows typical ITS1 amplification products obtained from *G. rostochiensis* and SP25, illustrating the difference in molecular weight.

Sequence alignment and primer design

A full characterisation of the sequences, including frequencies of nucleotide distribution and pairwise distances can be found in Chapter 4. The aligned ITS1 sequences from *G. rostochiensis* (AF016878), *G. pallida* (AF016871) and SP25 (DQ887561), with the locations of species-specific primers, can be seen in in Figure 6.1. Primers sagU1, sagR1 and sagP1 were used in a multiplex PCR to test its ability to distinguish between *G. rostochiensis, G. pallida* and SP25, particularly because cysts of the latter are being detected in samples from

potato fields in the Sandveld region on a regular basis. An amplification product of 575 bp was obtained when the PCR was performed with DNA from *G. rostochiensis* populations and a 403 bp product was amplified from *G. pallida* DNA. The primers were also able to detect both *G. rostochiensis* and *G. pallida* when mixtures of their DNA were introduced into a multiplex PCR reaction (Fig. 6.3). No amplification of *G. pallida* DNA took place with sagR1 and no amplification of *G. rostochiensis* DNA took place with sagP1. A 575 bp product was however also obtained from SP25 DNA on some occasions, indicating a possible false positive reaction with sagR1. Two new primers, sagS1 and sagR2 were designed to try and get rid of the false positive reaction from SP25. Primer sagS1 were designed to be specific to SP25 and consistently gave a PCR product of 231 bp when paired with sagU1 (Fig. 6.4). No false positives were amplified from any other species of *Globodera* with this primer. Primer sagR2 were designed to be specific for *G. rostochiensis* when paired with sagU1, giving a product of 311 bp. Unfortunately, extensive testing of this primer at different annealing temperatures also showed that a false positive is produced when DNA from SP25 is used in the reaction.

RESTRICTION FRAGMENT LENGTH POLYMORPHISMS

Three restriction enzymes, *Hpy8*I, *Rsa*I and *Xce*I were selected that were able to discriminate between *G. rostochiensis,* SP25, WK1 and SK18. The predicted restriction fragments resulting from *in silico* digestion of the same amplification fragment for these four species with *Alu*I, *Hpy8*I, *Rsa*I and *Xce*I are presented in Table 6.2. The fragments obtained from virtual digestion by *Rsa*I were confirmed by digestion of the amplification products as seen in Figure 6.5.

Discussion

Subbotin *et al.* (2011) reported that the *G. rostochiensis* specific primer developed by Bulman and Marshall (1997) was well matched with the corresponding sequence regions for the ITS of *G. artemisiae* and *G. millefolii*, potentially giving false-positive results with these samples, and warned against the application of such methods that did not take non-agriculturally important *Globodera* into consideration, to samples from unknown locations.

After considering the diversity of South American populations of *Globodera*, Grenier *et al.* (2010), emphasised that the development of new molecular tools should include an obligatory validation process on a defined *Globodera* species and set of populations before adoption as a reference test. An attempt was made here to design species-specific primers that would discriminate between quarantine and non-quarantine cyst nematode species in potato fields. In this study, a specific primer in the ITS1 region that will successfully distinguish *G. rostochiensis* from the indigenous *Globodera* species, SP25, has not being successfully accomplished. A false positive reaction was observed with two separate primers that were designed for *G. rostochiensis* specificity. We attribute this to the fact that sequence differences are not sufficient in the areas where quality primers for both species are located.

Digestion of the ITS region of rDNA with selected restriction enzymes are currently applied in many laboratories for the identification of various organisms, including cyst nematodes (Subbotin *et al.,* 2010). Our *in silico* analysis indicated that the PCR-RFLP technique could be used to discriminate between local *Globodera* spp. Diagnosis with RFLP patterns and species-specifc primers, however, is a direct result from differences in the sequence regions used, leading us to the conclusion that the study of these sequences would give the most

detailed information regarding the identity of the organism studied. Sufficient sequence diversity was observed in the ITS regions of these nematodes to be able to distinguish them from each other. Given the problems experienced with other techniques, the amplification of a targeted DNA region (in this case the ITS regions of rDNA), followed by direct sequencing of the PCR product is regarded as the most accurate method to distinguish between these nematodes, provided that the infrastructure and knowledge to perform this kind of test is available.

Literature

BATES. J.A., TAYLOR, E.J.A., GANS, P.T. & THOMAS, J.E. (2002). Determination of relative proportions of *Globodera* species in mixed populations of potato cyst nematodes using PCR product melting peak analysis. *Molecular Plant Pathology*. 3, 153-161.

BULMAN, S.R. & MARSHALL, J.W. (1997). Differentiation of Australasian potato cyst nematode (PCN) populations using the polymerase chain reaction (PCR). *New Zealand Journal of Crop and Horticultural Science* 25, 123-129.

CLAPP, J.P., VAN DER STOEL, C.D. & VAN DER PUTTEN, W.H. (2000). Rapid identification of cyst (*Heterodera* spp., *Globodera* spp.) and root-knot (*Meloidogyne* spp.) nematodes on the basis of ITS2 sequence variation detected by PCR-single-strand conformational polymorphism (PCR-SSCP) in cultures and field samples. *Molecular Ecology* 9, 1223-1232.

FERRIS, V.R., MILLER, L. I, FAGHIHI, J. & FERRIS, J.M. (1995). Ribosomal DNA comparisons of *Globodera* from two continents. *Journal of Nematology* 27, 273-283.

FLEMING, C.C., MOWAT, D.J. & POWERS, T.O. (1993). Potato cyst nematode diagnostics using the polymerase chain reaction. In: Ebbels, D. (ed.), *Proceedings of Plant Health and the European Single Market Symposium*. British Crop Protection Council, Farnham, UK, pp. 349-354.

FULLAONDO, A., BARRENA, E., VIRIBAY, M, BARRENA, I., SALAZAR, A & RITTER, E. (1999). Identification of potato cyst nematode species, *Globodera rostochiensis* and *G. pallida* by PCR using specific primer combinations. *Nematology* 1, 157-163.

GRENIER, E., FOURNET, S., PETIT, E. & ANTHOINE, G. (2010). A cyst nematode 'species factory' called the Andes. *Nematology* 12, 163-169.

KNOETZE, R., MALAN, A.P. & MOUTON, C. (2006). Differentiation of South African potato cyst nematodes (PCN) by analysis of the rDNA internal transcribed spacer region. *African Plant Protection* 12, 103–110.

MADANI, M., SUBBOTIN, S. & MOENS, M. (2005). Quantitative detection of the potato cyst nematode, *Globodera pallida* and the beet cyst nematode, *Heterodera schachtii*, using real-time PCR with SYBR green I dye. *Molecular and Cellular Probes*. 19, 81-86.

MADANI, M., WARD, L.J. & DE BOER, S.H. (2008). Multiplex real-time polymerase chain reaction for identifying potato cyst nematodes, *Globodera pallida* and *Globodera rostochiensis*, and the tobacco cyst nematode, *Globodera tabacum. Canadian Journal of Plant Pathology* 30, 554-564.

MADANI, M., WARD, L.J. & DE BOER, S.H. (2011). Hsp90 gene, an additional target for discrimination between the potato cyst nematodes, *Globodera rostochiensis* and *G.*

pallida, and the related species, *G. tabacum tabacum. European Journal of Plant Pathology* 130, 271-285.

MULHOLLAND, V., CARDE, L., O' DONNELL, K.J., FLEMING, C.C. & POWERS, T.O. (1996) Use of the polymerase chain reaction to discriminate potato cyst nematode at the species level. In: Marshall, G. (ed.), *Proceedings of Diagnostics in Crop Production Symposium*. British Crop Production Council, Farnham, UK, pp. 247-252.

NAKHLA, M.K., OWENS, H.J., LI, W., WEI, G., SKANTAR, A.M. & LEVY, L. (2010). Multiplex Real-Time PCR assays for the identification of the potato cyst and tobacco cyst nematodes. *Plant Disease* 94, 959-965.

NOWACZYK, K., DOBOSZ, R., KORNOBIS, S. & OBREPALSKA-STEPLOWSKA, A. (2008). TaqMan Real-Time PCR-based approach for differentiation between *Globodera rostochiensis* (golden nematode) and *Globodera artemisiae* species. *Parasitology Research* 103, 577–581.

POWERS, T.O. (2004). Nematode molecular diagnostics: From bands to barcodes. *Annual Review of Phytopathology* 42, 367–83.

POWERS, T.O. & HARRIS, T.S. (1993). A polymerase chain reaction method for identification of five major *Meloidogyne* species. *Journal of Nematology* 25, 1-6.

POWERS, T.O., TODD, T.C., BURNELL, A.M., MURRAY, P.C.B., FLEMING, C.C., SZALANSKY, A.L., ADAMS, B.A. & HARRIS, T.S. (1997). The rDNA internal transcribed spacer region as a taxonomic marker for nematodes. *Journal of Nematology* 29, 441-450.

PYLYPENKO, L.A, UEHARA, T., PHILLIPS, M. S., SIGAREVA, D.D. & BLOK, V.C. (2005). Identification of *Globodera rostochiensis* and *G. pallida* in the Ukraine by PCR. *European Journal of Plant Pathology* 111, 39-46.

QUADER, M., NAMBIAR, L. & CUNNINGTON, J. (2008). Conventional and real-time PCR-based species identification and diversity of potato cyst nematodes (*Globodera* spp.) from Victoria, Australia. *Nematology* 10(4), 471-478.

SHIELDS, R., FLEMING, C.C. & STRATFORD, R. (1996). Identification of potato cyst nematodes using the polymerase chain reaction. *Fundamental Applied Nematology* 19, 167-173.

ŠIRCA, S., UREK, G. & MEGLIČ, V. (2003). Molecular and Biochemical methods used for the identification of *Globodera* species in Slovenia. *Plant Protection Science* 39, 151-153.

SKANTAR, A.M., HANDOO, Z.A., CARTA, L.K. & CHITWOOD, D.J. (2007). Morphological and molecular identification of *Globodera pallida* associated with potato in Idaho. *Journal of Nematology* 39, 133-144.

SUBBOTIN, S.A., CID DEL PRADO VERA, I., MUNDO-OCAMPO, M. & BALDWIN, J.G. (2011). Identification, phylogeny and phylogeography of circumfenestrate cyst nematodes (Nematoda: Heteroderidae) as inferred from analysis of ITS-rDNA. *Nematology* 13, 805-824.

SUBBOTIN, S.A, MUNDO-OCAMPO, M. & BALDWIN, J.G. (2010). *Systematics of cyst nematodes (Nematoda: Heteroderidae).* Nematology monographs and perspectives Volume 8A. Brill, Leiden-Boston.

SZALANSKI, A.L., SUI, D.D., HARRIS, T.S. & POWERS, T.O. (1997). Identification of Cyst Nematodes of Agronomic and Regulatory Concern with PCR-RFLP of ITS1. *Journal of Nematology* 29, 255-267.

THIERY, M. & MUGNIERY, D. (1996). Interspecific rDNA restriction fragment length polymorphism in *Globodera* species, parasites of solanaceous plants. *Fundamental and Applied Nematology* 19, 471-479.

TOYOTA, K., SHIRAKASHI, T., SATO, E., WADA, S. & MIN, Y.Y. (2008). Development of a real-time PCR method for the potato-cyst nematode *Globodera rostochiensis* and the root-knot nematode *Meloidogyne incognita*. *Soil Science and Plant Nutrition* 54, 72–76.

UEHARA,T., KUSHIDA,A., ITOU,K., NARABU,T. & MOMOTA,Y. (2005). Discrimination of three cyst-forming nematodes of the genus *Globodera* (Nematode: Heteroderidae) from Japan based on PCR-RFLP of ribosomal DNA. *Applied Entomology And Zoology (Japan)* 40, 537-543.

VEJL, P., SKUPINOVÁ, S., SEDLÁK, P. & DOMKÁŘOVÁ, J. (2002). Identification of PCN species by using ITS1 region polymorphism. *Rostlinná Výroba* 48 (11), 486-489.

VRAIN, T. C., WAKARCHUCK, D. A., LÈVESQUE, A. C. & HAMILTON, R. I. (1992). Intraspecific rDNA restriction fragment length polymorphism in the *Xiphinema americanum* group. *Fundamental and Applied Nematology* 15, 563-573.

YU, H., CHRONIS, D., LU, S. & WANG, X. (2011). Chorismate mutase: an alternatively spliced parasitism gene and a diagnostic marker for three important *Globodera* nematode species. *European Journal of Plant Pathology* 129, 89-102.

Table 6.1: Primers used in this study.

Primer code	Description	Primer sequence
rDNA1	Forward primer for amplification of ITS1-5.8S-ITS2	5'-TTGATTACGTCCCTGCCCTTT-3'
rDNA2	Reverse primer for amplification of ITS1-5.8S-ITS2	5'-TTTCACTCGCCGTTACTAAGG-3'
rDNA1.58	Reverse primer for amplification of ITS1	5'-ACGAGCCGAGTGATCCACCG-3'
sagU1	Universal primer for species-specific amplification	5'-GATTACGTCCCTGCCCTTTG-3'
sagR1	Specific primer for *G. rostochiensis*	5'-CAAGCGCAGACATGCCGCAA-3'
sagR2	Specific primer for *G. rostochiensis*	5'-CAATGTGCCAGCCAATACAC-3'
sagP1	Specific primer for *G. pallida*	5'-CGACAACAGCAATCGTCGAG-3'
sagS1	Specific primer for SP25	5'-CAAACTGGACAGCAGGTAAC-3'
PunQF1	Internal sequencing primer	5'-GAACTGCAGAAACCTTGA-3'
PunQR1	Internal sequencing primer	5'-TTCACCTACAGCTACCTT-3'

Table 6.2. Approximate sizes (in bp) of restriction fragments generated by virtual digestion of the ITS1-rRNA regions of *Globodera* spp.

Species	Unrestricted fragment	*Alu*I	*Hpy8*I	*Rsa*I	*Xce*I
G. rostochiensis	754	80, 149, 163, 362	24, 100, 148, 482	9, 23, 203, 221, 298	27, 190, 257, 280
Globodera sp. SP25	766	163, 604	24, 28, 127, 148, 439	9, 23, 112, 112, 205, 305	58, 80, 121, 256, 260
Globodera sp. SK18	767	163, 603	17, 24, 28, 70, 127, 148, 439	4, 5, 23, 112, 211, 412	29, 71, 98, 282, 287
Globodera sp. WK1	761	162, 224, 375	24, 28, 67, 70, 103, 147	4, 5, 23, 206, 523	10, 61, 122, 281, 287

```
Gr: TTGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTGCCCGGGACTGAGCCATTTCGAGAAACTCG 70
Gp: ............................................................................ 70
Gc: ............................................................................ 70

Gr: GGGACGATTATGCGTGTCGGCTTCGGTCGTCGCGTTGATTGGAACCGATTTAATCGCAGTGGCTTGAACC 140
Gp: ............................................................................ 140
Gc: ...........................................A................................ 140

Gr: GGGCAAAAGTCGTAACAAGGTAGCTGTAGGTGAACCTGCTGCTGGATCATTACCCAAGTGATACCAATTC 210
Gp: ............................................................................ 210
Gc: ..........................................................-..... 209

Gr: ACCACCTACCTGCTGTCCAGTTGAGTCAGTGTGGGCAACACCACATGCCTCCGTTTGTTGTT-GACGGAC 279
Gp: ..........................................................-..... 279
Gc: ....GT.............TG.............T..........T..........T..... 279

Gr: -ACATGCCCGCTGTGTAT---TGGCTGGCACATTGACCAACAAT---GTACGGACAGCGCCCTGTGGGCA 342
Gp: -..........A...T.---G.............T.....---...T.............. 342
Gc: C.T.....T.GG....TTT.....................G.TGT...C.............. 349

Gr: CATGAGTGTTGGGGTGTAACCGATGTTGGTGGCCCTATGGGTGAGCCGACGATTGCTGCTGTCGTCGGGT 412
Gp: ................................A........CT...........T.......... 412
Gc: ..A....T.........T.T.A..........T..G........GC.........T...CA......... 419

Gr: CGCTGCGCCAACGGAGGAAGCACGCCCACAGGGCACCCGAACGGCTGTGCTGGCGTCTGTGCGTCGTTGA 482
Gp: ......A..........TG.......................................................... 482
Gc: .....TG...........A.T........................................................ 489

Gr: GCGGTTGTTGCGCCTTGCGCAGATATGCTAACATGGAGTGTAGGCTG--CTACTCCATGTTGTACGTGCC 549
Gp: ..................G........G.................--...T.......C......... 549
Gc: ..................T..C.....................TG...C.......T....A.A.. 559

Gr: GTACCTTGCGGCATGTCTGCGCTTGTGTGCTACGTCCGTGGCCGTGATGAGACGACGTGTTAGGACCCGT 619
Gp: .....CA..................................................................... 619
Gc: .....T...........G........................................................... 629

Gr: GCCTGGCATTGGCACGTGGTTTAAGACTTGATGAGTGCCCGCAGGCACCGCCAGC-TTTTTCCCATTTTT 688
Gp: ...............C.....................T.............-......T....... 688
Gc: ..............T.....................C.............G.............. 699

Gr: ATTTATTTTTT-AATGCAATTCGATTGCTAAAATATTCTAGTCTTATCGGTGGATCACTCGGCTCGT    754
Gp: ..AA.......-..GT.....T...GT.....................................    754
Gc: ..T.T......CC.TG.....C...TG.....................................    766
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Fig. 6.1. Alignment of the ribosomal internal transcribed spacer (ITS1) from *Globodera rostochiensis* Gr), *G. pallida* (Gp) and SP25 (Gc). Sequence differences are shown, (.) represents identical base and (-) represents deletions. Estimated positions of the 18S and 5.8S genes are indicated by shading. Specific primers for the different species as designed in this study are underlined.

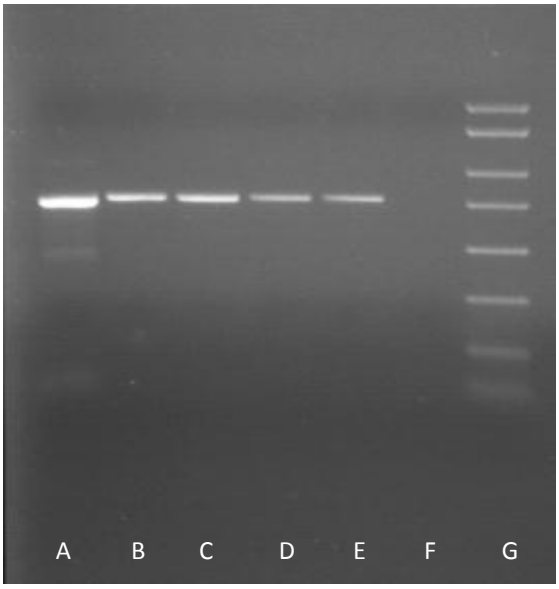

Fig. 6.2. ITS1 amplification products of *Globodera* spp. A: *G. rostochiensis*; B-E: SP25; F: Negative control; G: PCR marker (Sigma).

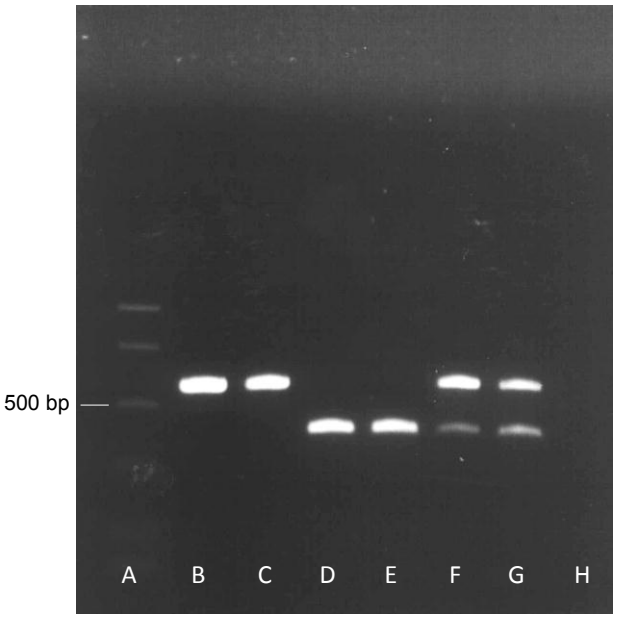

Fig. 6.3. Amplification products of *Globodera* spp. with primers sagU1, sagR1 and sagP1 in a multiplex PCR. B, C: *G. rostochiensis*; D, E: *G pallida*; F.G: a mixture of *G. pallida* and *G. rostochiensis;* H: Blank reaction. A: Molecular weight marker (Sigma).

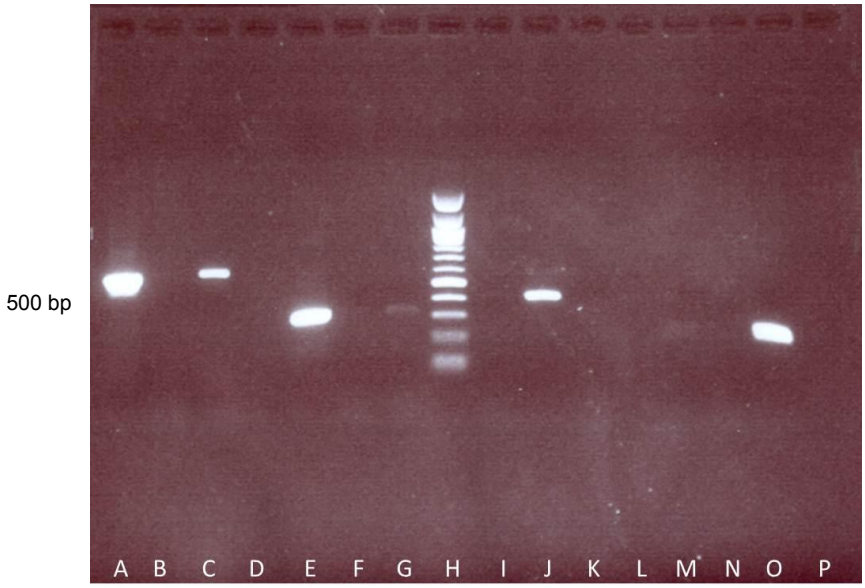

Fig. 6.4. Amplification products of *Globodera* spp. with different species-specific primers. A: *G. rostochiensis* with sagR1; B: *G. pallida* with sagR1; C: SP25 with sagR1; E: *G. rostochiensis* with sagR2; F: *G. pallida* with sagR2; G: SP25 with sagR2; I: *G. rostochiensis* with sagP1; J: *G. pallida* with sagP1; K: SP25 with sagP1; M: *G. rostochiensis* with sagS1, N: *G. pallida* with sagS1; O: SP25 with sagS1. D, L, P: Blank reactions. H: Molecular weight marker (Sigma).

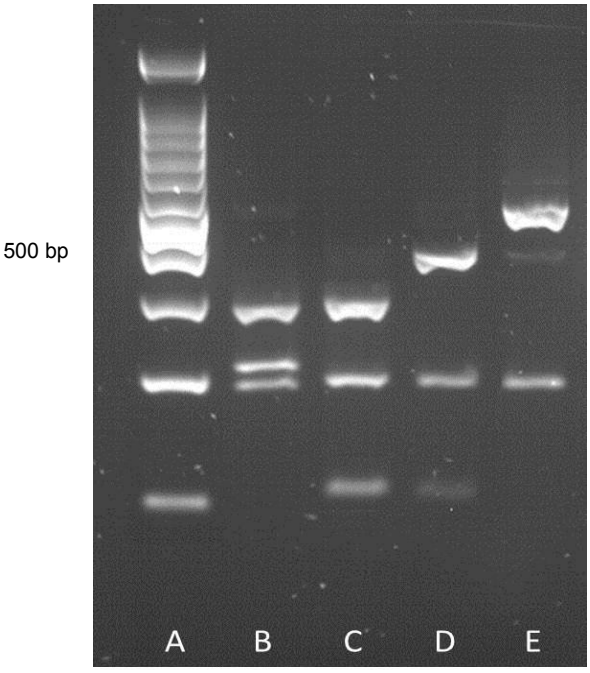

Fig. 6.5. *Rsa*I digestion products of the amplified ITS1 region of *Globodera* spp. B: *G. rostochiensis*; C: SP25; D: SK18; E: WK1; A: 100 bp marker (Fermentas).

Chapter 7

Comparative morphology of South African *Globodera* species (Nematoda: Heteroderidae) from different geographical regions in South Africa

Introduction

The life cycle of cyst nematodes comprises the egg, juveniles and adults. The active part of the life cycle starts when the second-stage juvenile (J2) hatches from the egg (Turner & Evans, 1998). The J2 is attracted to the growing root tip and enters the root by cutting through the cell walls with the stylet (Evans & Stone, 1977). At the third juvenile stage, females or males start to develop. Females become saccate and enlarge as their gonads increase in size. Males leave the root and are attracted to females that exude sex pheromones. After fertilisation, the embryos develop within the egg as far as the second juvenile stage, commonly called the J2, while still within the body of the female. The female then dies and transforms into a protective cyst containing 200-500 eggs (Turner & Evans, 1998).

The morphology of the cysts nematodes are complicated by their sexual dimorphism, sedentary lifestyle of the female and the presence of a dormant cyst. Several of the life stages are important in their morphology and useful for identification. The cysts and juveniles are of the greatest value and are the most widely used (Golden, 1986).

Globodera cysts exhibit relatively less growth in length than other cyst genera, resulting in a spherical shape. The shape of *Punctodera* cysts is usually pyriform, while *Heterodera* and *Cactodera* species form cysts with a posterior protuberance, the terminal or vulval cone

(Fleming & Powers, 1998). Cyst sizes and shapes are variable though, and identification of genera and species is more reliable when based on other characters.

The vulval cone, vulval basin and the perineal characteristics are very important in generic and species identifications. Membranous areas, called fenestrae, on the vulval cone or perineal region are composed of much thinner layers of tissue which are lost in older cysts and form escape exits for juveniles (Fleming & Powers, 1998). The vulva may be carried on a band of thickened tissue, the vulval bridge. Four main patterns of fenestration are recognised in the cyst nematodes: If two thin-walled areas or holes are present one on each side of the vulval bridge, the condition is called ambifenestrate when the fenestrae abut the vulval bridge or bifenestrate when the fenestrae are well separated and more or less circular (Siddiqi, 2000). A single thin-walled region or hole, without the vulva supported on a bridge of tissue, is termed circumfenestrate (Siddiqi, 2000). The position of the anus in respect to the vulva is used for generic and species differentiation. The presence of an extra fenestra around the anus is a major characteristic of the genus *Punctodera* (Siddiqi, 2000). The surface of the perineum shows ridges and furrows in two basic patterns (Hesling, 1978): (i) a reticulate or maze-like pattern and (ii) a pattern of roughly parallel ridges. The number and shapes of the cuticular ridges between the anus and the vulval basin, the vulval basin diameter and the distance from the anus to the vulval basin are all important for species identification (Fleming & Powers, 1998). In the genus *Heterodera* the remnants of tissues and muscles of the vaginal wall is attached to the cyst wall by two arms running across the vulval cone and called the underbridge. A well-developed underbridge is lacking in the genera *Globodera*, *Cactodera*, *Punctodera* and *Dolichodera*. Associated with the ends of the underbridge are knob-like structures, the bullae which are perhaps the remnants of the muscles (Siddiqi, 2000). In some cyst-forming species, adult females and newly formed cysts may be surrounded by a thick,

chalk-like deposit called the subcrystalline layer. Golden (1986) does not regard the subcrystalline layer to be of diagnostic value, because of its variability and brief existence.

Juveniles are second only to cysts in their importance in identification of cyst nematodes (Golden, 1986). The analysis of the juvenile stylet may permit separation of several species of *Globodera*. J2 stylet length is one of the more reliable juvenile characters which can be measured and used to separate *Globodera pallida* Stone, 1973 and *G. rostochiensis* (Wollenweber, 1923) Skarbiloviich, 1959 (Gonzalez *et al*., 1992). The shape of the stylet knobs is also useful, with some species exhibiting smaller rounded knobs while others generally has more robust, hooked knobs (Fleming & Powers, 1998). Labial patterns of the J2 basically consist of a labial disc surrounded by two lateral, two subdorsal and two subventral lips (Baldwin, 1992). In many cyst nematode species, this pattern is modified, often by fusion of adjacent submedial lips with one another and with the labial disc. Sometimes the submedial lips further fuse with the labial annuli. The labial pattern is also variable among species by the number of annuli on the head (Subbotin *et al.*, 2010). The J2 body length, as well as shape of the tail and the hyaline region may be useful in species diagnosis (Wouts & Baldwin, 1998). The lateral lines are also informative characters. Most species have four lines, but *Betulodera, Dolichodera* and some *Heterodera* species have three lines (Subbotin *et al.* 2010).

The males and females of cyst nematodes are more difficult to obtain and are not generally utilised in morphological studies and identification procedures. The morphological features of the female, including shape, size and posterior region are similar to those of the cysts, but length of the female stylet can be used in the differentiation of species. The male structures which are of morphological value include the length of the stylet, shape of the stylet knobs, lateral field and the morphology of the spicules (Golden, 1986).

The morphological identification of *Globodera* species is based on several morphological and morphometric characters. In addition to the morphological features described above there are measurements and ratios that are widely used for the descriptions of species. Some of these measurements and ratios for diagnosis of *Globodera* species according to Golden (1986), Siddiqi (2000) and Subbotin *et al.* (2010) are listed in Table 7.1. These diagnostic characters have relatively stable means for *Globodera* species and may be useful for identification, but in some populations the range may be extended by a few individuals (Subbotin *et al.*, 2010). Identification keys for *Globodera* are provided by Wouts (1984), Golden (1986), Wouts and Baldwin (1998) and Subbotin *et al.* (2010).

Scanning electron microscopy (SEM) has also proved to be a valuable tool in studying the morphology of cyst nematodes. It is especially useful for observing labial patterns and lateral lines of J2 and males, as well as the body wall and cone patterns of females and cysts (Mulvey & Golden, 1983; Othman *et al.*, 1988). Subbotin *et al.* (2010) provides an overview of methods for the application of electron microscopy for the diagnosis of cyst nematodes.

Morphological characterisation is still regarded as the essential basis for the diagnosis of nematodes. Several geographic populations of *Globodera rostochiensis* have been identified in South Africa. In previous chapters, genetic differences have been shown to exist between them. Phylogenetic analyses of molecular sequence data has also identified the existence of different isolates of indigenous cysts, clearly differentiated from known sequences of other *Globodera* species.

In this study, morphological descriptions and morphometric measurements of five geographical populations of *G. rostochiensis*, as well as for four populations of indigenous cysts will be compared.

Materials and Methods

SOURCES OF ISOLATES

Isolates from indigenous populations of cysts were collected in the Cape Floristic Kingdom of South Africa (Chapter 2). Since sequences from the cysts collected from fallow potato fields in the Sandveld region were almost identical, (Chapter 2), only one isolate, SP25, was selected for inclusion. As it was morphologically evident that a population of lemon-shaped cysts, with a terminal cone (OK 14), as described in Chapter 2, was not one of the circumfenestrate cyst nematodes, it was not considered for inclusion in this analysis. For ease of reference the isolates will be referred to as SP25, SK18, WK1 and WK2.

G. rostochiensis in South Africa is represented here by five geographically separate populations. They originate from unique locations in the Sandveld, Ceres, South-Western Cape, Eastern Cape and Gauteng potato-producing areas. For ease of reference they will henceforth be called the Sandveld, Ceres, Philippi, Hankey and Gauteng populations. All morphological and morphometric data for *G. rostochiensis* included in this chapter are derived from these populations.

EXTRACTION OF NEMATODES

Cysts and females were extracted from soil with the aid of a Seinhorst cyst elutriator (Seinhorst, 1964). Infective juveniles (J2) were obtained from opened cysts. Males were extracted from soil using Oostenbrink's elutriation and sieving method (Oostenbrink, 1960).

LIGHT MICROSCOPY

Whole cysts were soaked overnight in distilled water, after which the posterior end of the cyst was cut off with an ocular scalpel, cleaned, trimmed and then transferred to 90% ethanol. The cuticle piece (terminal pattern) was cleared in Euparal Essence and mounted in a drop of Euparal on a glass slide. A cover slip was applied and sealed with glyceel. J2 and males were fixed in FPG (Netcher & Seinhorst, 1969), dehydrated to anhydrous glycerin by using the short Seinhorst (1959) method and permanently mounted in anhydrous glycerin on glass slides. Females were placed in cold lactophenol and incubated at 40° C for 3 days. The female body was then cut in half in lactic acid on a glass slide and then transferred to a drop of glycerol on a glass slide, where the body contents was removed and the cuticle trimmed around the vulval area. The vulval area and head of the female was then permanently mounted in anhydrous glycerin on a glass slide. Photomicrographs of cyst vulval cones, females, males and J2 were taken with a Optikam PRO 3 Digital Camera (Optika microscopes, Ponteranica, Italy) attached to a Nikon Eclipse 80i (Nikon Instruments Inc., Melville, NY) with DIC (differential interference contrast). Measurements were made using the Optika Vision Pro Software (v. 2.7).

SCANNING ELECTRON MICROSCOPY

For scanning electron microscopy (SEM), juveniles were ether recovered from cysts, fixed with TAF or removed from microscope slides, dehydrated in an ethanol series, critical point dried, mounted on microscope stubs and coated with gold-palladium (21 nm). For external

and internal morphology of the cysts, the techniques described by Lax and Doucet (2002) were followed, after which they were mounted on microscope stubs and coated with gold-palladium (21 nm).

Results

Unless otherwise stated, all morphological descriptions of *G. rostochiensis* followed the description of Subbotin *et al*. (2010). Morphometric data of all the populations are given in Tables 7.1 – 7.6.

FEMALES

(Fig. 7.1)

Since the hosts of the indigenous cysts are not known, no females of these populations were found. Therefore, only females of *G. rostochiensis* are studied here. The females of *G. rostochiensis* are pearly white, subspherical to ovate, with elongate, protruding necks. Their colour changes from white to yellow to light golden as the female matures to the cyst stage. They have thick cuticles, with superficial, rugose, lace-like patterns. A D-layer is present, with punctations resolved near or beneath the surface. The labial region is slightly offset, bearing two annuli. The labial framework is weakly developed. The stylet is fairly strong, straight to slightly curved, with well developed rounded basal knobs, sloping posteriorly. Median bulb large, nearly spherical, with well developed valve. Excretory pore conspicuous, always at or near base of neck. Vulva terminal, with a slit of medium length. Vulval area

circumfenestrate. No anal fenestration present, but the anus and vulva both lie in a 'vulval basin'.

The morphemetrical data of the females from the different populations of *G. rostochiensis* are shown in Table 8.1. Females from the Gauteng population of *G. rostochiensis* were generally larger in size than females from the other populations, while females from the Hankey populations were the smallest. The females measured from all the populations were also larger than the sizes for females published by Golden & Ellington (1972) (Average length including neck = 0.63 mm vs. 0.52 mm; average width = 0.43 mm vs. 0.34 mm). The L/W ratio of females from the SA populations was also lower for than the published data from Golden & Ellington (1972) (1.1 vs 1.5), which indicates that the cysts measured by Golden & Ellington (1972) were more ovate than those measured for the South African populations. The average vulval basin diameter of females from the SA populations were smaller than the diameter reported by Stone (1973b) (17.7 µm vs. 22.4 µm). Females from the SA populations had less cuticular ridges on average between the anus and vulva as was reported by Stone (1973b) (19.0 vs. 21.6).

CYSTS

(Figs. 7.2, 7.5, 7.7, 7.9, 7.11)

The cysts from all the species were light brown to dark brown in colour, but some yellow cysts were observed from SP25. Cysts from *G. rostochiensis*, SP25, SK18 and WK1 were ovate to spherical in shape, but an obtuse terminal cone were observed in specimens from WK2. The perineal region of all the cysts were circumfenestrate. The anus of *G.*

rostochiensis, SP25, SK18 and WK1 were positioned at the apex of a V-shaped subsurface mark in cuticle of some specimens. No V-shaped subsurface mark was observed in cuticle of WK2. The anus of *G. rostochiensis* was generally more conspicuous than that of the other populations. Bullae or vulval bodies were observed as large, variously shaped knobs in SP25, WK1 and WK2, but not in *G. rostochiensis* and SK18. Cuticular ridges on the outer surface of cysts between the anus and vulval basin were observed in all specimens, but in *G. rostochiensis* the lines were more numerous (14 -25), when compared to the indigenous cysts (6 – 14). In most specimens, these ridges changed to irregular patterns in area beyond vulval basin and around and just beyond anus, and again to crescentic wavy ridges, extending to neck-area, but in WK2 specimens the ridges become irregular again in the vicinity of the excretory pore. On the vulval cone of WK2 specimens, parallel vertical lines, connecting with lines formed by cuticular ridges at right angles, were observed.

Cysts from the Hankey population of *G. rostochiensis* were generally smaller in size than cysts from the other populations (see Table 8.2 for measurements), but cysts from the Philippi population also exhibited shorter body widths, resulting in a higher L/W ratio than the other cysts. Cysts from the SA populations were also smaller than most published data, but comparable to measurements from Pakistan (Subbotin *et al.*, 2010). The cysts from the Philippi population exhibited much more variation in their distance from anus to vulval basin than the other populations (36.9 µm to 117.6 µm), resulting in the highest Granek's ratio (3.9 ± 1.0) of the SA populations. The distance from the vulval basin to anus of the indigenous cysts was significantly less than that of *G. rostochienis*, resulting in lower Granek's ratio's (1.6 – 2.0 vs. 2.7 – 3.9) for all the indigenous specimens (see Table 8.2 for measurements).

MALES

(Fig. 7.1)

Only males of *G. rostochiensis* were observed. Prominent annulations were observed on the cuticle. The labial region are slightly offset, hemispherical, with six annuli. The labial framework is heavily sclerotised. Stylet strong, with prominent knobs. Lateral fields with four equally spaced lines were observed. The median bulb was ellipsoidal in shape. The spicules were slightly arcuate with rounded tips.

Tail lengths of males from the Ceres, Philippi, Hankey and Gauteng populations of *G. rostochiensis* were slightly longer than those published by Golden & Ellington (1972), resulting in a lower c ratio (267 vs. 186), except for the Sandveld population, were these values were much closer to the published data. The average spicule length of the Gauteng population were shorter on average than those of the other populations, which were consistent with the published data. (Golden & Ellington, 1972; Stone, 1973b).

SECOND-STAGE JUVENILES

(Figs. 7.3, 7.4, 7.6, 7.8, 7.10, 7.12)

The bodies of all specimens observed were curved slightly ventrally, with the tail tapering to finely rounded terminus. In the case of SK18, the terminus was more finely pointed than for the other populations. In all specimens the head was slightly offset from the rest of the body. The number of head annuli varied from three for SP25, SK18 and WK1 to four for *G. rostochiensis* and as many as seven for some specimens of WK2. With SEM, the medial lips

and labial disc of SP25 were oval-rectangular in face view, raised above the lateral lips. The prestoma opening was rectangular, slightly raised above rest of labial disc and medial lips. The lateral lips were large, rounded, bearing amphid apertures; separating first labial annule to form two medial crescents. The labial patterns for SK18 and WK1 were similar, but the lateral lips were more rectangular, sometimes irregular in shape for SK18 and in WK1 the submedial lips were fused with one another and with the labial disc in some specimens. The lip region of WK2 exhibited a hexaradiate appearance with SEM. The labial disc were circular in face view (SEM), consisting of six triangular plates, each one bearing a labial papillae. The prestoma opening was oval, slightly raised above rest of labial disc and medial lips. The medial lips and lateral lips were fused, rectangular in appearance, bearing amphid apertures and four outer labial sensilla; partially fused with and separating first labial annule to form six medial crescents. The stylets of all the populations were well developed for all populations, with the cone occupying less than 50 % of total stylet length. The shape of the stylet knobs varied from rounded (*G. rostochiensis*, SP25, SK18 and WK1); flattened (SP25, SK18 and WK1) to anchor-shaped (WK2). The average length of the stylets varied from 21 µm for *G. rostochiensis* to as much as 27 µm for WK1 (see Tables 8.4 and 8.6 for measurements).

DGO (dorsal gland outlet to stylet knobs) varied from 3.5 to 7.5 µm (see Tables 8.4 and 8.6). In all the specimens, the genital primordium were located slightly posterior to mid-body. In some specimens of WK2, a vulva located posterior from midbody (SEM) and functional gonads (LM) was observed. In most specimens, the nerve ring was situated a short distance posterior to median bulb with excretory pore at around a body width posterior of the nerve ring, but the nerve ring was indistinct in WK1 specimens. The median bulb valve was prominent in most specimens and ellipsoidal in form. Lateral fields with four incisures,

extending from about six annules posterior of labial region to 15 – 20 µm from tail tip (SEM). Incisures of *G. rostochiensis*, SP25, SK18 and WK1 were crenated, but not those of WK2. Areolation of the outer lines were observed in SP25, SK18 and WK1, but not in WK2. Tail terminus annulated to tip (SEM), varies from relatively smooth (SK18) to irregular in appearance (WK2).

Most of the characters measured for the J2's of the different populations of *G. rostochiensis* (Table 8.4) did not differ greatly between the populations, except for tail length where the Philippi population had slightly shorter tails than the rest, differing by 3-5 µm. However all the measurements for the J2's of *G. rostochiensis* were consistent with the data published in Subbotin *et al.* (2010), both in average and range.

Discussion

Characterisation and phylogenetic analyses of sequences from the isolates SP25, SK18 and WK1 (Chapter 4) confirmed their membership of the genus *Globodera*, and indicated that they are unique, previously undescribed, species in this genus. For SP25 and SK18, it is doubtful if the morphological and morphometrical data alone are sufficient to determine if these isolate constitute new species of cyst nematodes, but for WK1 the morphological differences are arguably sufficient to regard WK1 as a new species on the basis of the morphology. The combination of molecular and morphological data provided convincing evidence to to describe these three isolates as new species of *Globodera.*

Phylogenetic analyses of the sequence from WK2 clusters this nematode in a group with *Heterodera*, but the morphology of these specimens are not consistent with that of the

Heterodera genus. Following the key to the genera of Heteroderinae in Subbotin *et al.* (2010), these specimens are morphologically similar to *Betulodera*, in having a spheroid cyst with an obtuse vulval cone, thick cyst wall with an irregular network-like pattern and J2 with three incisures in the lateral field, but the the distance from vulval basin to anus is less than that of *B. betulae* (42.4 µm vs 81.0 µm), resulting in a lower Granek's ratio (1.76 vs 3.8) (Hirschmann & Riggs, 1969). The unique labial patterns of WK2 juveniles, as revealed by SEM, as well as the presence of a vulva and functional gonads in some of the juvenile specimens, sets this cyst nematode apart from both genera. The possibility exists that these specimens constitute a new genus of cyst nematodes, which needs to be carefully studied and described to substantiate the existing data. The unique character of some juveniles showing a functional reproductive system may even point to an ancestral adult stage.

Analysis of data from RAPD banding patterns presented in Chapter 5, suggests that the Sandveld population of *Globodera rostochiensis* is genetically different from the other populations within the species. However these genetic differences was not expressed in the morphological features studied in this chapter. No significant differences between the morphology and morphometrics of five different populations of *G. rostochiensis* could be determined. The fact that the females measured in this study was generally larger than the published data, can be attributed to the fact that females in different developmental stages was used in the different studies. The fact that the females from Golden & Ellington (1972) were more oval than round, supports this. The fact that cysts from the South African populations were also smaller than most published data, but comparable to measurements from Pakistan (Subbotin *et al.*, 2010) can be attributed to the fact that these populations all originate from warmer climates, influencing the development of females and cysts. Other methods for the determination of intraspecific variations between these populations,

including pathotype tests, hatching tests and reproduction tests will be employed in upcoming chapters to further characterise these populations.

Literature

BALDWIN, J.G. (1992). Evolution of cyst and non-cyst forming Heteroderinae. *Annual review of Phytopathology* 30, 271-290.

EVANS, K. & STONE, A. R. (1977). A review of the distribution and biology of the potato cyst nematodes *Globodera rostochiensis* and *G. pallida*. *PANS* 23, 178-189.

FLEMING, C.C. & POWERS, T.O. (1998). Potato cyst nematode diagnostics: morphology, differential hosts and biochemical techniques. In: Marks, R.J. & Brodie, B.B. (Eds.). *Potato Cyst Nematodes. Biology, Distribution and Control.* Wallingford, UK, CAB International, , pp.91 – 114.

GOLDEN, A. M. (1986). Morphology and identification of cyst nematodes. In: Lamberti, F. & Taylor, C. E. (Eds.) *Cyst Nematodes*. New York, USA, Plenum Press, pp. 23-79.

GOLDEN, A.M. & ELLINGTON, D.M.S. (1972). Redescription of *Heterodera rostochiensis* (Nematoda: Heteroderidae) with a key and notes on closely related species. *Proceedings of the Helminthological Society of Washington* 39, 64-77.

GONZALEZ, J.A., PHILLIPS, M.S. & HACKETT, C.A. (1992). Morphological variability of potato cyst nematodes (*Globodera* spp.) in the Canary Islands. *Annals of Applied Biology* 120, 315-328.

HIRSCHMANN, H & RIGGS, R.D. (1969). *Heterodera betulae* n. sp. (Heteroderidae), a cyst-forming nematode from river birch. *Journal of Nematology* 1, 169–179.

HESLING, J.J. (1978). Cyst nematodes: morphology and identification of *Heterodera*, *Globodera* and *Punctodera*. In: Southey, J.F. (ed.) *Plant Nematology*. Her Majesty's Stationary Office, London, pp 125-171.

LAX, P. & DOUCET, M.E. (2002). New process for preparing heteroderid cysts for scanning electron microscopy. *Nematology* 4, 445 – 447.

MULVEY, R.H. & GOLDEN, A.M. (1983). An illustrated key to the cyst-forming genera and species of Heteroderidae in the western hemisphere with species morphometrics and distribution. *Journal of Nematology* 15, 1-59.

NETSCHER, C. & SEINHORST, J.W. (1969). Propionic acid better than acetic acid for killing nematodes. *Nematologica* 15, 286.

OOSTENBRINK, M. (1960). Estimating nematode populations by some selected methods. In: Sasser, J.N. and Jenkins, W.R. (eds.) *Nematology*. University of North Carolina Press, Chapel Hill. pp. 85-102.

OTHMAN, A.A., BALDWIN, J.G. & MUNDO-OCAMPO, M. (1988). Comparative morphology of *Globodera*, *Cactodera* and *Punctodera* spp. (Heteroderidae) with scanning electron microscopy. *Revue de Nématologie* 11, 53-63.

SEINHORST, J.W. (1959). A rapid method for the transfer of nematodes from fixative to anhydrous glycerin. *Nematologica* 4, 67-69.

SEINHORST, J.W. (1964). Methods for the extraction of *Heterodera* cysts from not previously dried soil samples. *Nematologica* 10, 87-94.

SIDDIQI, M. R. (2000). *Tylenchida parasites of plants and insects*, 2nd edition. Wallingford, UK, CABI Publishing, xiii + 833 pp.

SKARBILOVICH, T.S. (1959) On the structure of the systematics of nematodes of the order Tylenchida Thorne, 1949. *Acta Parasitologica Polonica* 7, 117-132.STONE, A.R. (1973a) *Heterodera rostochiensis. CIH Descriptions of plant-parasitic nematodes,* Set 2, No 16. St. Albans, UK, Commonwealth Institute of Helminthology, 3 pp.

STONE, A.R. (1973b). *Heterodera pallida*. *CIH Descriptions of plant parasitic nematodes.* Set 2. No. 17. Farnham Royal, UK, Commonwealth Agricultural Bureaux.

SUBBOTIN, S.A, MUNDO-OCAMPO, M. & BALDWIN, J.G. (2010). *Systematics of cyst nematodes (Nematoda: Heteroderidae).* Nematology monographs and perspectives Volume 8A. Brill, Leiden-Boston, xii + 351 pp.

TURNER, S.J. & EVANS, K. (1998) The origins, global distribution and biology of potato cyst nematodes (*Globodera rostochiensis* (Woll.) and *Globodera pallida* Stone). In: Marks, R.J. & Brodie, B.B. (Eds.). *Potato Cyst Nematodes. Biology, distribution and control.* CAB International, Wallingford, Oxon OX10 8DE, UK, pp. 7 - 26.

WOLLENWEBER, H.W. (1923). Krankheiten und Beschädigungen der Kartoffel. *Arbeiten des Forschungsinstitutes für Kartoffelbau*, Heft 7, Paul Parey, Berlin, 56 pp.

WOUTS, W. M. (1984). *Globodera zelandica* n.sp. (Nematoda: Heteroderidae) from New Zealand, with a key to the species of the genus *Globodera*. *New Zealand Journal of Zoology* 11, 129-135.

WOUTS, W.M. & BALDWIN, J.G. (1998). Taxonomy and identification. In: Sharma, S.B. (Ed.). *The Cyst Nematodes*. Dordrecht, Boston & London, Kluwer Academic Publishers, pp. 83-122.

Table 7.1: Morphometric measurements and ratios used in the diagnosis of *Globodera* spp.

Life stage	Measurement / ratio	Symbol
Cyst and female	Body length	L
	Body width	W
	Body length / body width	L/W
	Vulval basin diameter	-
	Vulval basin to anus	-
	Distance from the edge of the vulval basin to the anus / diameter of the vulval basin	Granek's ratio (G)
Males and J2	Body length	L
	Body length / greatest body diameter	a
	Body length / distance from anterior end to junction of pharynx and intestine	b
	Body length / tail length	c
	Tail length / body diameter at anus	c'
	Distance from the base of the stylet to the dorsal gland opening	DGO

Table 7.2. Morphometrical data of female *Globodera rostochiensis* from different geographical areas in South Africa (All measurements in µm).

Characters	Sandveld population	Ceres population	Philippi population	Hankey population	Gauteng population
n	10	10	10	10	10
Length, excluding neck (L)	514.1 ± 46.2 (401.4-580.3)	496.2 ± 61.7 (411.5-593.5)	480.7 ± 25.1 (424.7-519.2)	403.4 ± 48.3 (311.8-471.6)	538.9 ± 49.7 (442.1-621.0)
Width (W)	447.0 ± 49.0 (336.1-511.6)	438.5 ± 76.6 (333.3-559.0)	445.7 ± 48.8 (377.5-534.2)	347.9 ± 60.5 (230.8-412.3)	497.4 ± 73.9 (406.6-597.3)
L/W	1.2 ± 0.1 (1.0-1.3)	1.1 ± 0.1 (1.0-1.5)	1.1 ± 0.1 (1.0-1.2)	1.2 ± 0.1 (1.0-1.4)	1.1 ± 0.1 (0.9-1.3)
Neck	149.3 ± 17.9 (109.1-170.5)	163.1 ± 30.4 (128.3-211.8)	140.9 ± 14.4 (124.9-169.8)	140.5 ± 23.8 (104.2-185.0)	153.1 ± 20.3 (105.8-174.9)
Stylet	21.6 ± 1.7 (17.5-23.6)	21.4 ± 1.5 (17.8-23.2)	21.1 ± 1.2 (19.1-23.2)	23.0 ± 1.4 (21.3-25.9)	21.7 ± 2.0 (17.8-24.5)
DGO	6.5 ± 1.1 (4.5-7.9)	6.5 ± 1.0 (5.2-8.6)	6.6 ± 0.8 (5.6-8.5)	6.4 ± 0.9 (5.2-7.9)	6.1 ± 0.9 (5.1-8.0)
Anterior end to median bulb valve	74.3 ± 16.5 (49.1-107.1)	74.5 ± 11.5 (59.9-97.5)	67.7 ± 11.6 (53.8-92.6)	77.9 ± 12.3 (59.5-100.6)	73.6 ± 8.3 (55.1-82.2)
Vulval basin diameter	17.6 ± 1.2 (15.5-20.1)	17.5 ± 2.4 (13.3-21.8)	17.2 ± 2.6 (12.5-23.6)	17.9 ± 2.0 (14.2-19.8)	18.3 ± 2.3 (15.3-21.5)
Vulval slit	9.7 ± 1.2 (8.2-11.9)	8.9 ± 1.3 (6.5-10.8)	9.8 ± 1.2 (7.1-11.4)	10.1 ± 0.5 (9.0-10.7)	10.0 ± 0.9 (8.2-11.1)
Vulval basin to anus	63.4 ± 13.7 (46.9-86.4)	57.3 ± 11.5 (38.3-81.8)	69.8 ± 14.6 (48.4-98.4)	59.3 ± 12.0 (39.8-86.1)	66.9 ± 13.1 (52.3-93.0)
Granek's ratio (G)	3.6 ± 0.8 (2.5-5.1)	3.3 ± 0.5 (2.8-4.4)	4.1 ± 0.9 (2.8-5.8)	3.4 ± 0.8 (2.1-4.5)	3.7 ± 0.9 (2.5-5.8)
Number of cuticular ridges between vulval basin and anus	18.3 ± 2.5 (15-24)	18.5 ± 2.0 (15-22)	20.2 ± 2.8 (15-25)	19.3 ± 2.9 (14-24)	18.8 ± 1.8 (15-21)

Table 7.3. Morphometrical data of cysts and eggs of *Globodera rostochiensis* (All measurements in µm).

Characters	Sandveld population	Ceres population	Philippi population	Hankey population	Gauteng population
Cysts (n)	10	10	10	10	10
Length, excluding neck (L)	520.9 ± 85.2 (409.2-645.9)	536.1 ± 62.8 (408.7-610.0)	503.3 ± 90.2 (380.2-635.5)	481.3 ± 52.6 (382.5-573.2)	524.5 ± 58.2 (433.9-613.3)
Width (W)	502.2 ± 95.3 (372.4-688.9)	522.2 ± 94.8 (341.9-653.0)	484.6 ± 134.7 (279.6-735.4)	484.6 ± 56.1 (368.0-580.2)	533.3 ± 85.4 (414.8-685.7)
L/W	1.0 ± 0.1 (0.9-1.1)	1.0 ± 0.1 (0.9-1.2)	1.1 ± 0.1 (0.8-1.4)	1.0 ± 0.1 (0.9-1.1)	1.0 ± 0.1 (0.9-1.1)
Neck	140.2 ± 17.9 (119.0-174.1)	117.9 ± 24.3 (89.7-169.0)	130.0 ± 27.7 (104.3-208.0)	105.1 ± 28.0 (72.7-160.5)	122.4 ± 16.7 (90.6-151.1)
Vulval basin diameter	16.9 ± 2.5 (12.7-21.3)	18.3 ± 4.6 (9.8-25.3)	17.9 ± 2.5 (13.2-22.0)	18.6 ± 2.8 (14.9-23.9)	18.4 ± 1.8 (15.0-20.4)
Vulval basin to anus	60.2 ± 8.2 (41.3-72.0)	63.5 ± 12.4 (41.9-84.4)	71.6 ± 24.1 (36.9-117.6)	50.6 ± 9.2 (32.9-59.4)	60.1 ± 12.3 (46.1-84.8)
Granek's ratio (G)	3.6 ± 0.7 (2.9-5.2)	3.7 ± 1.1 (2.6-6.9)	3.9 ± 1.0 (2.3-5.9)	2.7 ± 0.6 (1.9-3.7)	3.3 ± 0.6 (2.5-4.6)
Number of cuticular ridges between vulval basin and anus	17.1 ± 1.6 (15-21)	17.8 ± 2.4 (14-22)	19.2 ± 3.3 (15-25)	17.9 ± 2.2 (14-21)	19.3 ± 2.2 (15-23)
Eggs (n)	10	10	10	10	10
L	103.2 ± 3.8 (96.4-109.9)	102.8 ± 4.9 (94.8-112.4)	100.8 ± 4.0 (94.3-108.7)	103.9 ± 4.7 (94.0-108.7)	106.4 ± 3.8 (98.1-111.9)
W	43.7 ± 3.1 (39.0-50.1)	43.4 ± 2.2 (40.5-46.7)	44.5 ± 2.6 (39.3-49.3)	43.4 ± 1.7 (40.9-47.1)	43.0 ± 2.0 (39.7-46.2)
L/W	2.4 ± 0.2 (1.9-2.7)	2.4 ± 0.1 (2.2-2.6)	2.3 ± 0.1 (2.1-2.5)	2.4 ± 0.1 (2.2-2.7)	2.5 ± 0.1 (2.3-2.7)

Table 7.4. Morphometrical data of males of *Globodera rostochiensis* from different geographical areas in South Africa (All measurements in µm).

Characters	Sandveld population	Ceres population	Philippi population	Hankey population	Gauteng population
n	10	10	10	10	10
Body length (L)	1063.4 ± 27.5 (1014.6-1100.9)	1097.8 ± 38.5 (1057.4-1169.5)	1124.4 ± 62.2 (1028.8-1201.7)	1081.0 ± 60.1 (992.2-1157.8)	1091.4 ± 34.6 (1011.6-1130.7)
a	32.7 ± 2.1 (30.0-36.4)	34.4 ± 3.2 (28.6-39.4)	37.8 ± 3.5 (33.1-43.4)	33.5 ± 3.2 (29.0-38.6)	34.8 ± 2.4 (31.4-38.4)
b	7.1 ± 0.9 (5.5-8.5)	7.3 ± 0.7 (6.2-8.2)	6.4 ± 0.6 (5.2-7.0)	6.5 ± 0.6 (5.5-7.6)	7.0 ± 0.6 (5.5-7.8)
c	230.3 ± 35.1 (172.3-290.3)	184.6 ± 21.3 (145.9-210.7)	180.1 ± 18.5 (152.6-204.2)	192.4 ± 30.5 (146.6-247.6)	186.2 ± 18.1 (158.7-220.8)
c'	0.4 ± 0.03 (0.3-0.4)	0.4 ± 0.03 (0.4-0.5)	0.5 ± 0.06 (0.3-0.6)	0.4 ± 0.04 (0.4-0.5)	0.4 ± 0.03 (0.4-0.5)
Total stylet length	25.2 ± 0.9 (23.3-26.6)	24.2 ± 1.1 (21.2-25.4)	24.2 ± 0.9 (22.7-25.6)	24.9 ± 0.9 (23.3-26.5)	24.1 ± 1.1 (21.5-25.6)
DGO	5.7 ± 0.5 (5.1-6.6)	6.0 ± 1.0 (4.8-8.2)	5.8 ± 1.1 (4.3-8.4)	5.9 ± 1.2 (4.3-9.1)	5.7 ± 0.8 (4.8-7.4)
Anterior end to median bulb valve	92.9 ± 5.6 (81.1-99.5)	88.0 ± 9.0 (71.8-101.1)	93.6 ± 4.7 (83.8-100.9)	87.5 ± 5.3 (75.3-95.4)	86.3 ± 6.3 (72.5-95.2)
Anterior end to excretory pore	158.9 ± 9.3 (143.4-175.0)	164.3 ± 11.8 (151.6-184.0)	168.2 ± 9.3 (148.9-183.8)	159.0 ± 12.9 (145.0-180.8)	162.9 ± 14.9 (138.7-182.5)
Body width	31.2 ± 2.0 (27.8-34.4)	32.1 ± 2.5 (29.6-37.9)	29.9 ± 2.3 (25.1-32.9)	32.4 ± 2.1 (29.3-35.6)	31.7 ± 2.3 (29.1-35.3)
Tail length	4.7 ± 0.7 (3.3-6.1)	6.0 ± 0.8 (5.1-7.3)	6.3 ± 0.8 (5.4-7.7)	5.7 ± 0.7 (4.2-6.8)	5.9 ± 0.5 (5.1-6.8)
Tail width	12.5 ± 1.3 (10.7-15.5)	14.7 ± 1.4 (12.9-17.9)	13.6 ± 1.4 (11.8-17.0)	13.9 ± 1.6 (11.2-16.5)	13.5 ± 1.2 (11.9-16.2)
Spicules	35.3 ± 2.8 (31.8-41.4)	32.3 ± 1.8 (30.1-35.4)	31.0 ± 1.8 (27.3-33.9)	33.7 ± 2.7 (29.3-38.4)	29.5 ± 1.8 (26.5-33.0)
Gubernaculum	12.2 ± 0.7 (11.5-12.8)	-	-	12.0 ± 0.7 (10.7-12.9)	12.3 ± 0.5 (11.6-12.7)

Table 7.5. Morphometrical data of second stage juveniles of *Globodera rostochiensis* from different geographical areas in South Africa (All measurements in µm).

Characters	Sandveld population	Ceres population	Philippi population	Hankey population	Gauteng population
n	10	10	10	10	10
Body length (L)	436.6 ± 16.5 (412.0-465.4)	441.8 ± 19.2 (413.8-471.9)	420.1 ± 27.5 (373.0-467.7)	430.5 ± 19.3 (404.5-474.3)	435.8 ± 14.7 (407.5-459.4)
a	23.8 ± 0.9 (22.0-25.0)	23.2 ± 2.2 (19.8-27.5)	22.3 ± 1.9 (19.8-26.5)	23.9 ± 1.0 (22.7-26.4)	23.7 ± 0.8 (22.7-25.4)
b	2.9 ± 0.2 (2.7-3.5)	2.9 ± 0.2 (2.6-3.5)	2.8 ± 0.2 (2.5-3.0)	2.8 ± 0.3 (2.5-3.7)	3.1 ± 0.3 (2.6-3.4)
c	9.3 ± 0.5 (8.5-10.2)	9.0 ± 0.7 (7.4-10.0)	9.4 ± 0.4 (8.8-10.2)	8.9 ± 0.2 (8.8-9.2)	9.2 ± 0.7 (8.3-10.6)
c'	4.2 ± 0.4 (3.4-5.1)	4.2 ± 0.2 (3.9-4.6)	4.0 ± 0.2 (3.7-4.4)	4.2 ± 0.2 (4.0-4.7)	4.2 ± 0.3 (3.7-4.9)
Total stylet length	21.8 ± 0.9 (19.4-22.4)	21.6 ± 1.0 (19.7-23.2)	21.2 ± 0.7 (19.5-22.0)	21.6 ± 0.9 (20.2-23.3)	21.4 ± 0.6 (20.3-22.4)
Head width	9.0 ± 0.4 (8.5-9.6)	9.1 ± 0.5 (8.0-9.7)	9.1 ± 0.6 (8.2-9.8)	8.9 ± 0.5 (7.8-9.6)	9.2 ± 0.3 (8.6-9.9)
Head height	3.2 ± 0.3 (2.9-3.8)	3.4 ± 0.4 (2.6-4.2)	3.5 ± 0.5 (2.8-4.4)	3.4 ± 0.4 (2.9-4.5)	3.5 ± 0.2 (3.0-3.8)
DGO	5.1 ± 0.5 (4.2-5.8)	5.1 ± 0.4 (4.6-6.1)	4.9 ± 0.7 (3.5-5.8)	5.1 ± 0.8 (3.9-7.0)	5.7 ± 0.8 (3.8-6.8)
Anterior end to median bulb valve	64.8 ± 3.5 (59.2-70.5)	61.3 ± 3.2 (56.2-66.0)	61.5 ± 5.6 (54.6-71.4)	62.4 ± 3.8 (54.4-68.4)	63.0 ± 3.3 (57.9-66.6)
Anterior end to excretory pore	93.4 ± 4.8 (86.0-101.4)	90.8 ± 6.3 (79.7-101.1)	91.2 ± 8.3 (76.4-103.1)	91.8 ± 4.0 (86.8-101.1)	90.9 ± 4.8 (81.7-97.6)
Body width	18.4 ± 0.9 (17.1-20.3)	19.1 ± 1.3 (16.8-21.5)	18.8 ± 0.9 (17.5-20.7)	18.0 ± 0.7 (16.5-19.1)	18.4 ± 0.7 (17.2-20.0)
Tail length	47.0 ± 2.4 (43.7-52.1)	49.3 ± 4.7 (43.8-59.0)	44.8 ± 3.6 (39.3-50.3)	48.1 ± 2.0 (45.0-51.6)	47.6 ± 4.1 (41.0-54.1)
Tail width	11.2 ± 1.2 (9.0-13.7)	11.6 ± 0.9 (10.6-13.8)	11.1 ± 0.9 (9.7-12.8)	11.4 ± 0.5 (10.6-12.1)	11.2 ± 0.6 (10.3-12.2)
Length of hyaline tail region	22.7 ± 2.7 (19.0-27.9)	24.2 ± 1.8 (21.3-27.7)	22.9 ± 1.3 (20.2-24.5)	24.7 ± 3.5 (18.8-29.3)	22.8 ± 1.5 (20.0-24.3)

Table 7.6. Morphometrical data of cysts and eggs of indigenous cyst populations from different geographical areas in South Africa (All measurements in µm).

Characters	SP25		SK-18		WK-01		WK-02	
	n	Mean ± SD (range)	n	Mean ± SD (range)	n	Mean ± SD (range)	n	Mean ± SD (range)
Cysts								
Length, excluding neck (L)	11	457.0 ± 67.7 (365.0-580.0)	23	416.9 ± 46.5 (348.3-526.3)	16	549.0 ± 72.8 (405.2-706.8)	21	469.6 ± 87.1 (316.5-656.1)
Width (W)	11	390.0 ± 77.5 (330.0-470.0)	23	379.2 ± 47.8 (304.9-505.7)	16	486.0 ± 107.4 (266.4-643.8)	21	434.6 ± 78.9 (273.3-587.5)
L/W	11	1.2 ± 0.1 (1.0- 1.3)	23	1.1 ± 0.1 (1.0-1.3)	16	1.2 ± 0.2 (1.0-1.5)	21	1.1 ± 0.1 (1.0-1.2)
Neck	11	133.3 ± 20.7 (100.0-150.0)	9	76.9 ± 11.5 (62.3-98.2)	12	100.0 ± 29.1 (63.6-157.2)	16	91.1 ± 18.6 (68.5-129.6)
Vulval basin diameter	11	19.7 ± 3.1 (16.0-25.0)	23	17.6 ± 4.0 (11.7-26.1)	22	21.0 ± 3.1 (15.6-25.0)	18	25.0 ± 3.6 (18.5-32.5)
Vulval basin to anus	11	37.6 ± 10.2 (27.0-60.0)	21	28.6 ± 6.8 (19.1-47.0)	22	33.6 ± 8.8 (19.9-53.5)	16	42.4 ± 6.6 (31.5-54.9)
Granek's ratio (G)	11	2.0 ± 0.8 (1.3-4.0)	21	1.7 ± 0.5 (1.0-3.0)	22	1.6 ± 0.4 (1.0-2.5)	16	1.7 ± 0.3 (1.2-2.2)
Number of cuticular ridges between vulval basin and anus	11	12.6 ± 3.5 (8-20)	21	7.2 ± 1.4 (6-12)	16	10.6 ± 1.7 (8-14)	14	9.6 ± 1.6 (7-12)
Eggs								
L	10	90 ± 7.2 (77.7-99.9)	11	107.6 ± 5.1 (95.6-113.2)	10	134.3 ± 8.8 (116.6-147.6)	6	114.5 ± 3.4 (108.9-119.3)
W	10	39.4 ± 4.44(33.3-44.0)	11	44.0 ± 2.5 (40.6-49.8)	10	49.2 ± 2.8 (44.0-54.2)	6	48.9 ± 2.0 (46.0-51.7)
L/W	10	2.3	11	2.5 ± 0.2 (2.1-2.7)	10	2.7 ± 0.3 (2.3-3.1)	6	2.3 ± 0.1 (2.1-2.5)

Table 7.7. Morphometrical data of second stage juveniles of indigenous cyst populations from different geographical areas in South Africa (All measurements in µm).

Characters	SP25		SK-18		WK-01		WK-02	
	n	Mean ± SD (range)	n	Mean ± SD (range)	n	Mean ± SD (range)	n	Mean ± SD (range)
Body length (L)	16	494.6 ± 24.6 (448.0-528.0)	23	465.0 ± 25.8 (416.2-523.5)	20	580.1 ± 25.3 (545.2-630.2)	9	452.3 ± 27.4 (398.1-491.3)
a	16	24.6 ± 1.6 (21.9-26.8)	20	25.3 ± 2.0 (21.1-29.2)	20	29.4 ± 1.7 (26.7-32.8)	9	22.8 ± 1.8 (19.9-25.3)
b	16	3.4 ± 0.4 (2.7-3.7)	20	2.9 ± 0.2 (2.5-3.4)	20	3.4 ± 0.5 (2.7-4.4)	8	2.7 ± 0.2 (2.5-3.1)
c	16	9.1 ± 0.9 (6.4-10.2)	20	8.4 ± 0.5 (7.6-9.4)	20	9.1 ± 0.4 (8.2-9.8)	9	8.9 ± 0.5 (8.3-10.0)
c'	16	4.1 ± 0.4 (3.4-5.2)	20	4.5 ± 0.3 (3.9-5.3)	20	4.7 ± 0.3 (4.3-5.4)	9	4.0 ± 0.3 (3.5-4.4)
Total stylet length	16	26.3 ± 1.4 (24.0-28.0)	20	23.5 ± 0.7 (22.5-24.8)	20	27.0 ± 0.9 (25.3-28.5)	9	24.0 ± 2.3 (18.6-25.8)
Head width	16	9.3 ± 0.6 (8.0-10.0)	20	8.7 ± 0.3 (8.2-9.3)	20	9.6 ± 0.4 (8.7-10.3)	9	9.0 ± 0.2 (8.6-9.4)
Head height	16	3.9 ± 0.2 (3.5-4.0)	20	3.4 ± 0.3 (3.0-4.1)	20	3.5 ± 0.3 (3.1-4.1)	9	3.4 ± 0.3 (2.8-3.8)
DGO	16	5.3 ± 0.7 (4.0-6.5)	20	4.4 ± 0.7 (3.5-6.5)	20	5.6 ± 0.7 (4.6-7.2)	7	6.2 ± 0.7 (5.3-7.4)
Anterior end to median bulb valve	16	64.9 ± 6.9 (53.0-70.0)	20	67.8 ± 4.8 (59.7-76.8)	20	73.5 ± 6.8 (62.9-87.1)	7	70.5 ± 5.7 (60.3-77.7)
Anterior end to excretory pore	16	99.0 ± 8.6 (79.0-110.0)	20	93.9 ± 7.2 (78.2-107.1)	20	103.1 ± 7.3 (88.4-119.0)	7	102.0 ± 6.5 (93.3-109.3)
Body width	16	20.1 ± 1.1 (19.0-23.0)	20	18.4 ± 0.8 (17.3-19.8)	20	19.7 ± 1.0 (17.7-21.3)	9	19.9 ± 1.0 (18.4-22.0)
Tail length	16	54.6 ± 5.8 (48.0-73.0)	20	55.7 ± 3.3 (48.8-63.9)	20	63.9 ± 3.8 (57.3-76.8)	9	51.1 ± 3.7 (45.1-55.5)
Tail width	16	13.2 ± 1.0 (12.0-15.0)	20	12.3 ± 0.7 (11.1-13.8)	20	13.5 ± 0.7 (12.1-15.0)	9	12.9 ± 0.7 (11.6-14.3)
Length of hyaline tail region	16	29.5 ± 2.9 (24.0-35.0)	20	24.9 ± 2.6 (18.7-29.3)	20	31.6 ± 3.7 (21.6-38.8)	9	28.9 ± 2.6 (24.6-32.7)

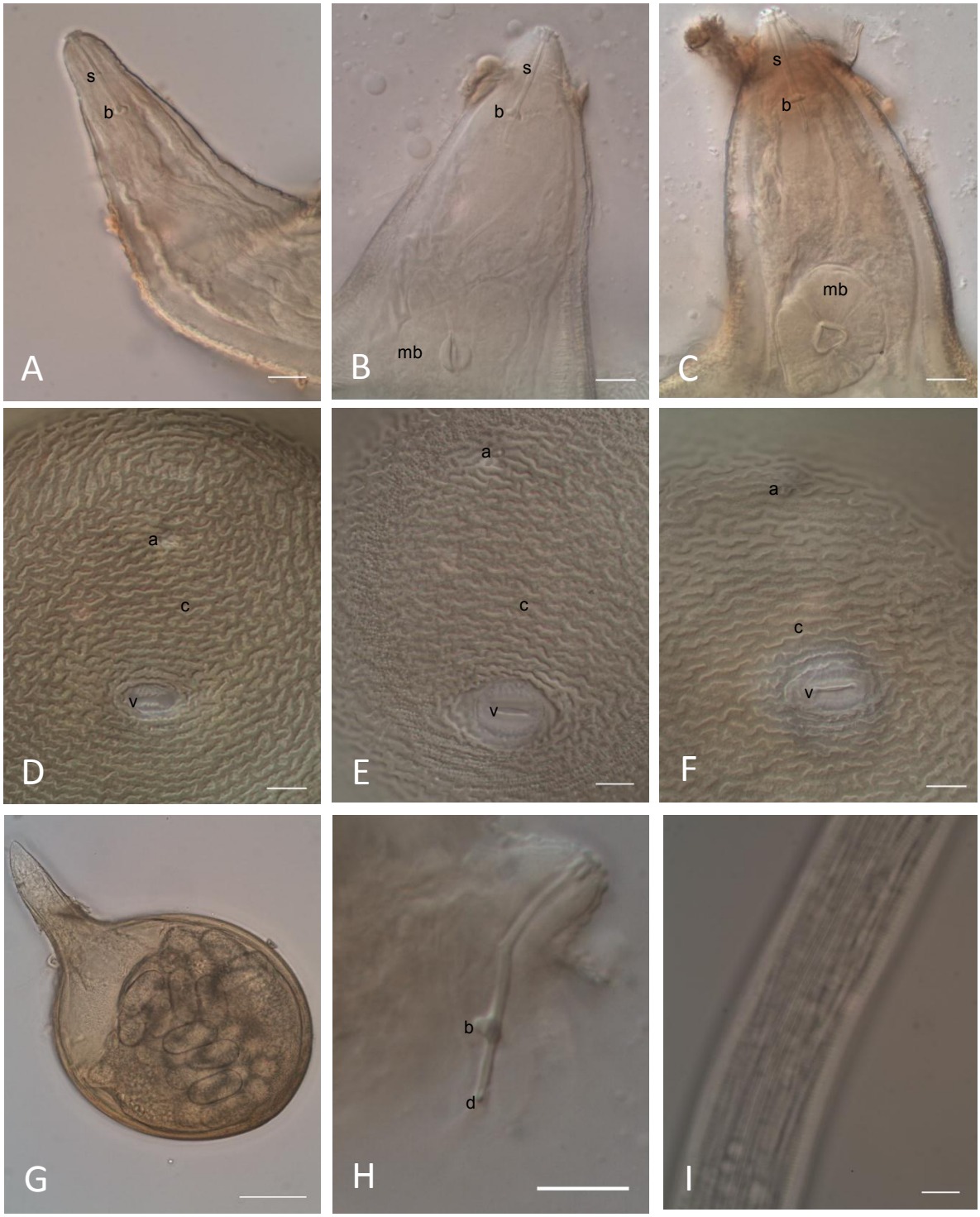

Fig. 7.1. Photomicrographs of females and males of *Globodera rostochiensis* from South Africa. A, B, C: Anterior region with s = stylet, k = knobs, mb = median bulb; D, E, F: Anal-vulval regions with v = vulval slit, a = anus and c = cuticular ridges; G: Whole female; H: Dissected stylet with k = knobs and d = dorsal oesophageal gland outlet; I: Male body showing lateral lines. (Scale bars: A-F,H,I = 10µm; G = 100 µm).

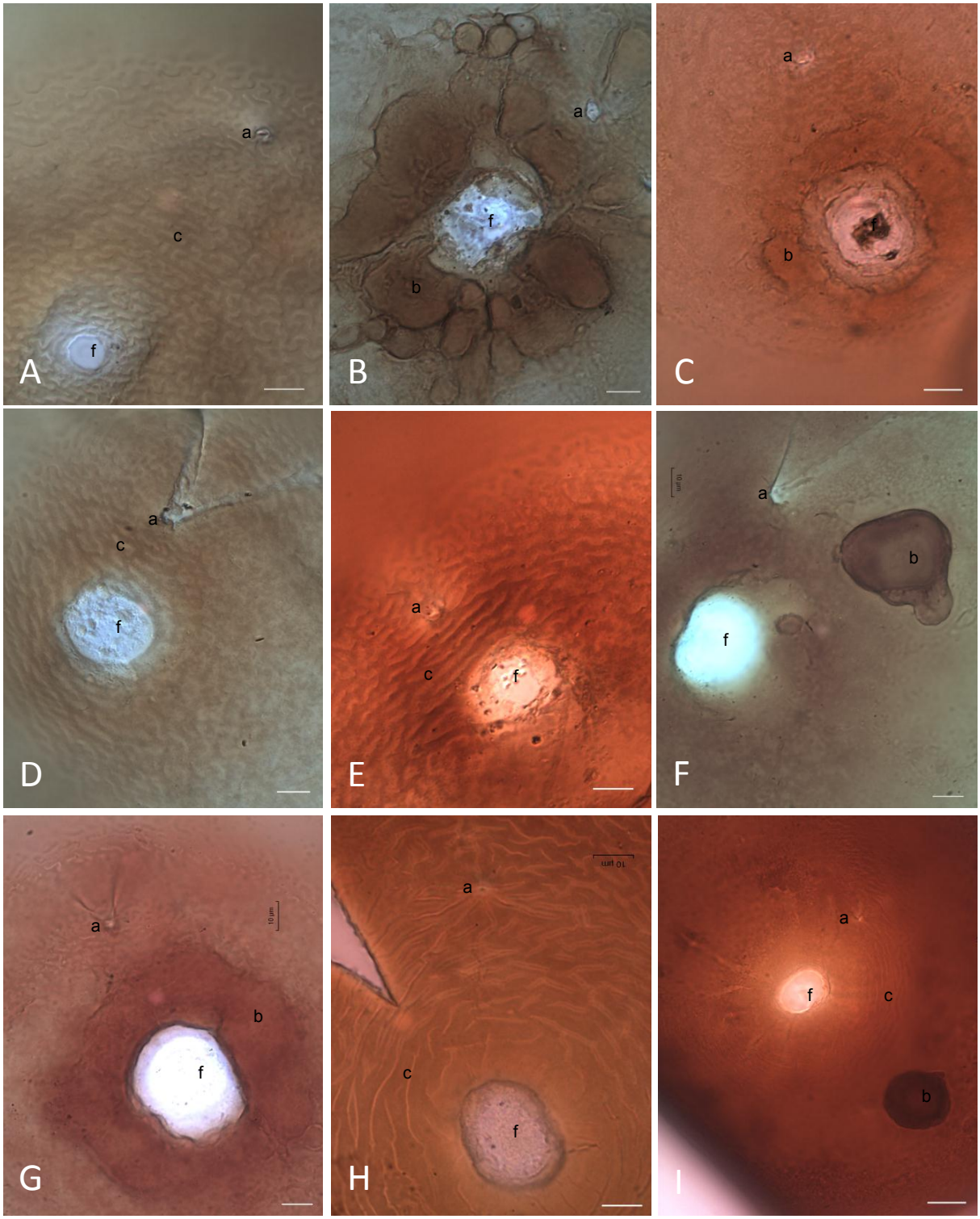

Fig. 7.2. Photomicrographs of cysts from South Africa. A**:** *Globodera rostochiensis*; B, C:SP25*;* D, E: SK18; F, G: WK1; H, I: WK2. For all figures a = anus, c = cuticular ridges, f = vulval fenestra and b = vulval bodies / bullae. (Scale bars = 10µm)

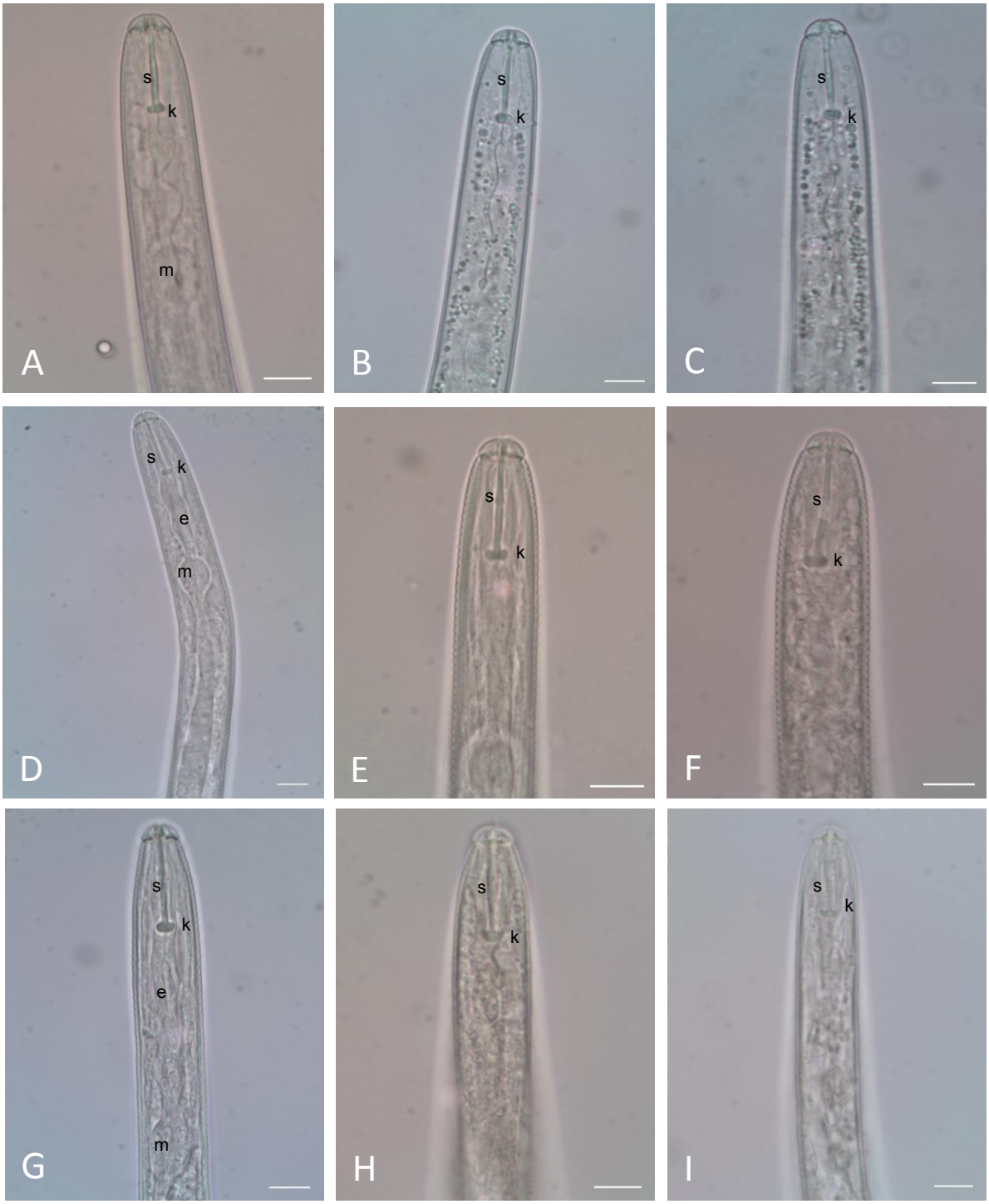

Fig. 7.3. Photomicrographs of anterior regions of second-stage juveniles of cyst nematodes from South Africa: A: *Globodera rostochiensis;* B, C: SP25*;* D, E: SK18; F, G: WK1; H, I: WK2. For all figures s = stylet, k = knobs, e = oesophagus and m = median bulb. (Scale bars = 10µm)

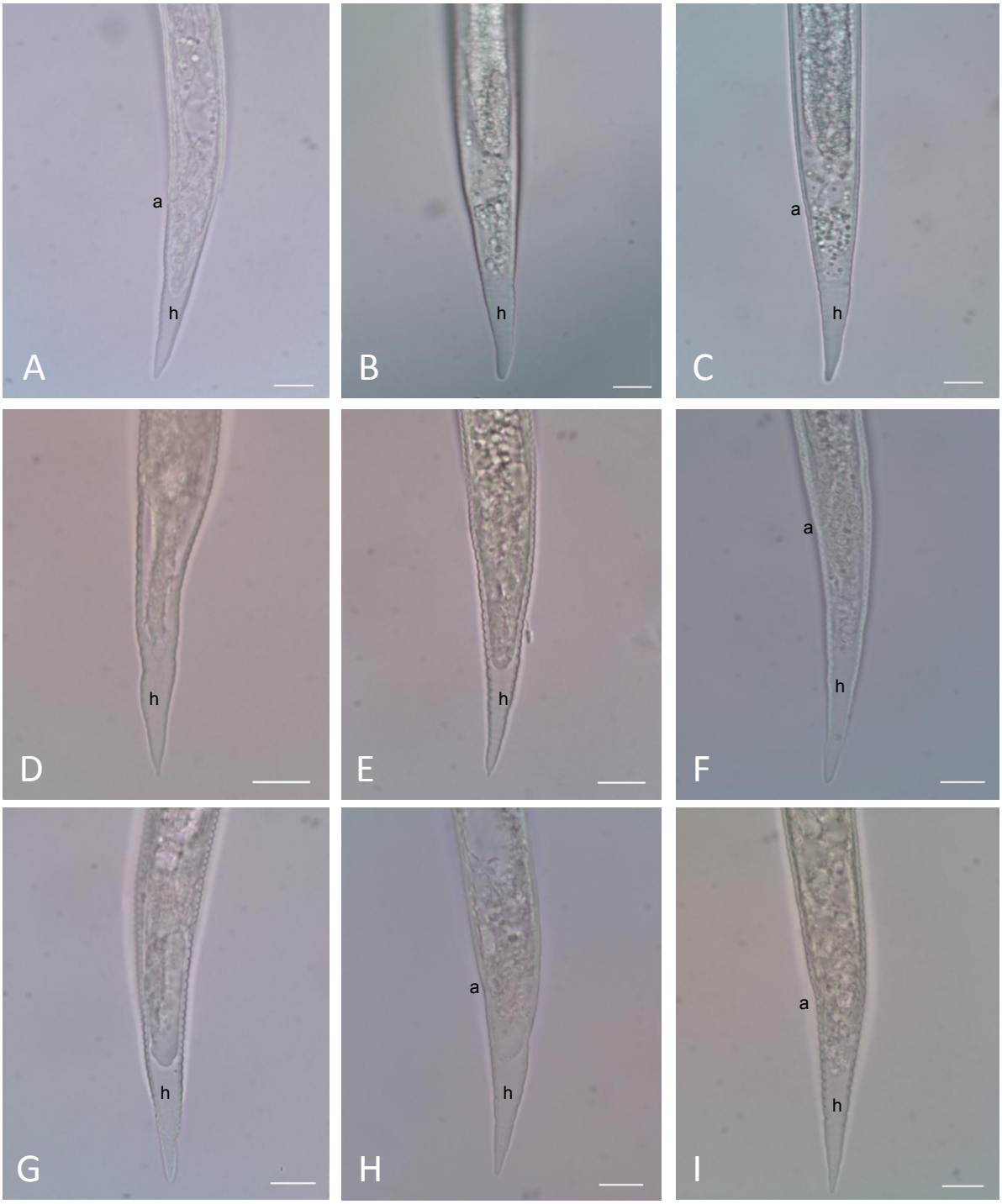

Fig. 7.4. Photomicrographs of tail regions of second-stage juveniles of cyst nematodes from South Africa: A: *Globodera rostochiensis;* B, C: SP25; D, E: SK18; F, G: WK1; H, I: WK2. For all figures a = anus and h = hyaline region. (Scale bars = 10µm)

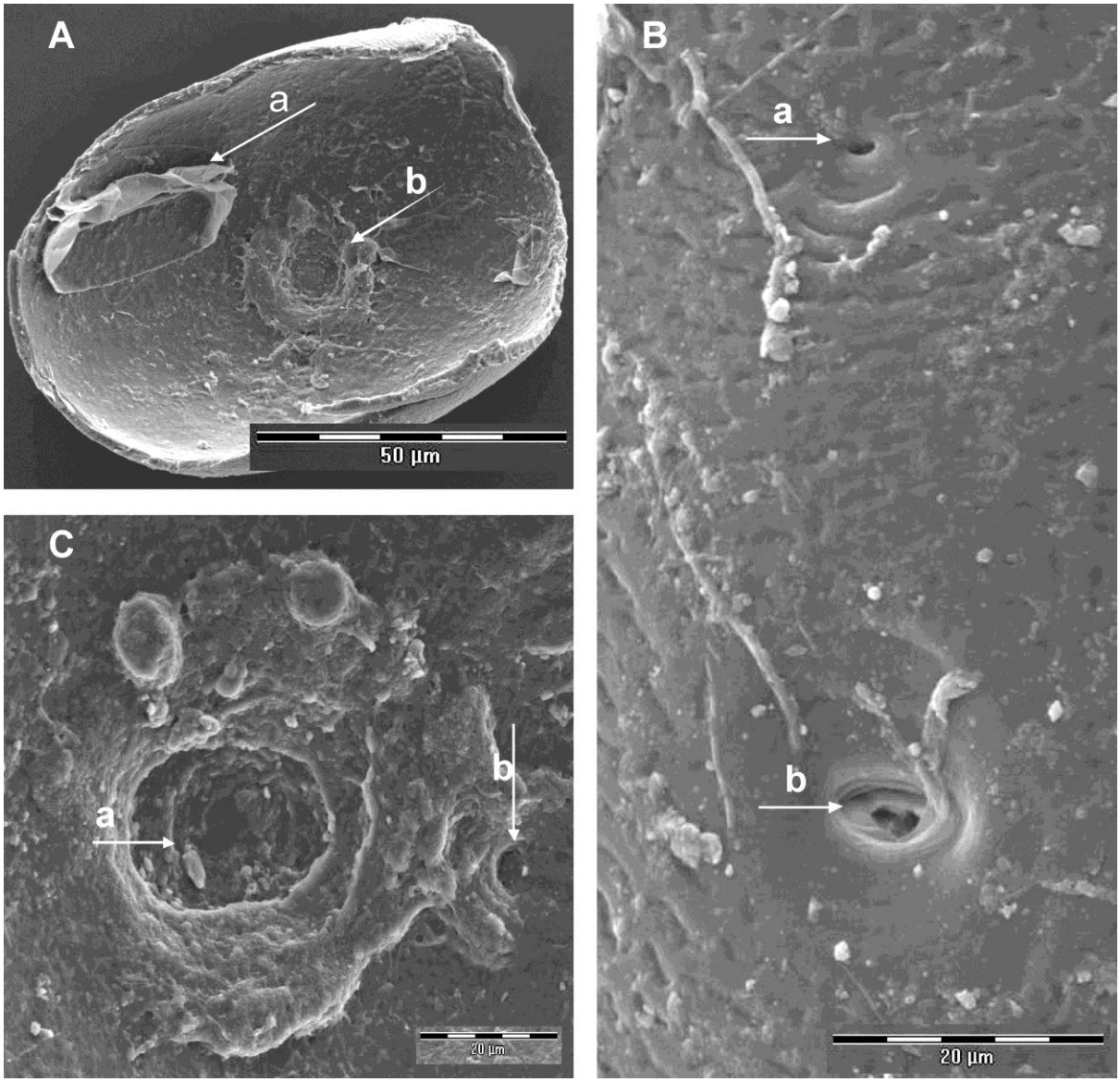

Fig. 7.5. Cysts of population SP25 (SEM). A: Internal morphology of the cyst wall (arrow a = an oval shaped body of unknown origin; arrow b = cuticular thickenings and bullae in the vulval basin – anus area); B: External morphology of the cyst wall (arrow a = anus b = vulval basin); C: Internal morphology of the cyst wall, showing the bullae and cuticular thickenings around the vulval basin (arrow a) and the anus (arrow b).

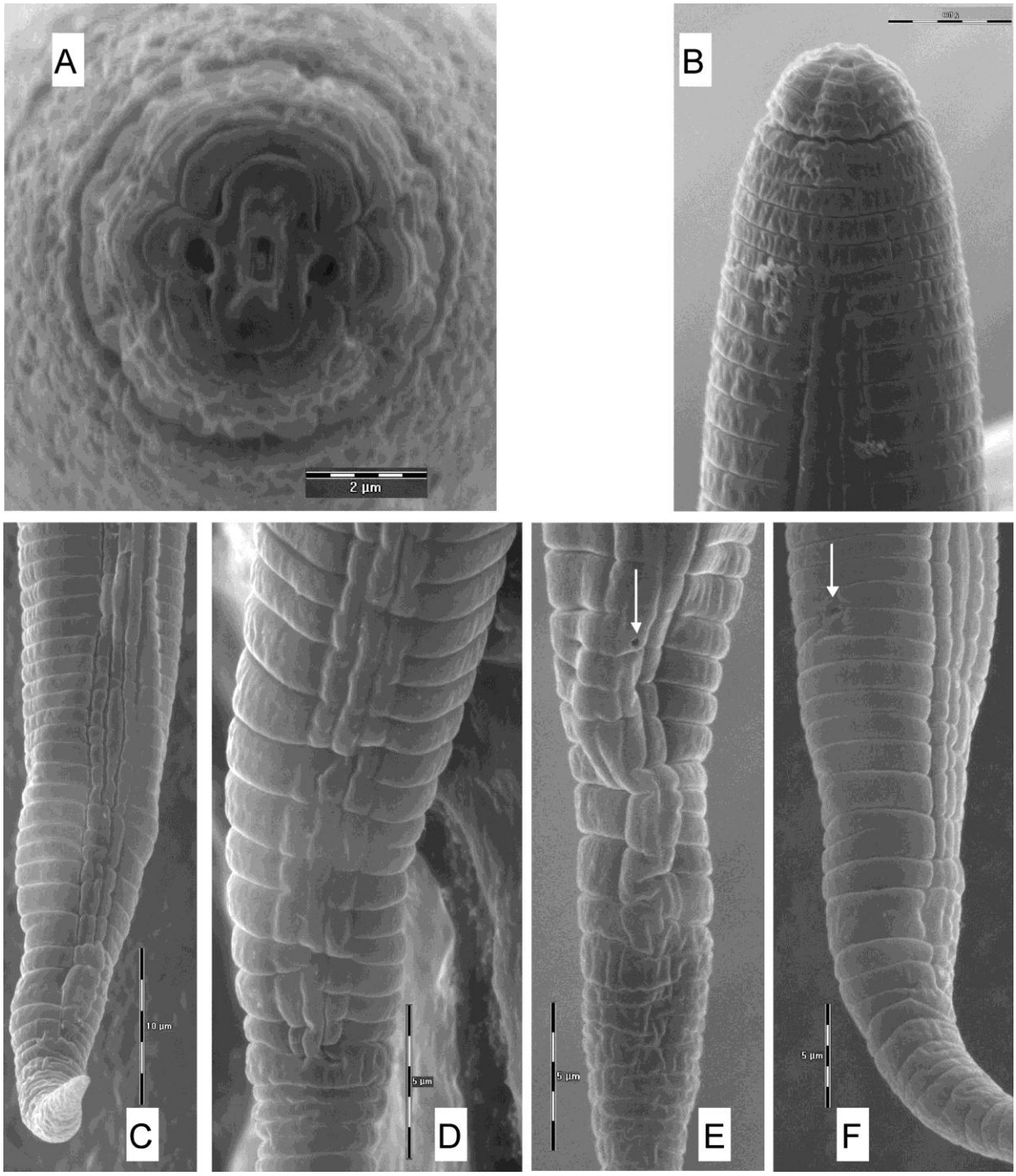

Fig. 7.6. Second stage juveniles (J2) of population SP25 (SEM). A, B: Head (A: Frontal view; B: Lateral view); C, D, E, F: Tail (C: Lateral view of whole tail; D – E: Lateral view of the ending of lateral lines, arrow showing the phasmid; F: Ventral view of the tail area, arrow showing the anus).

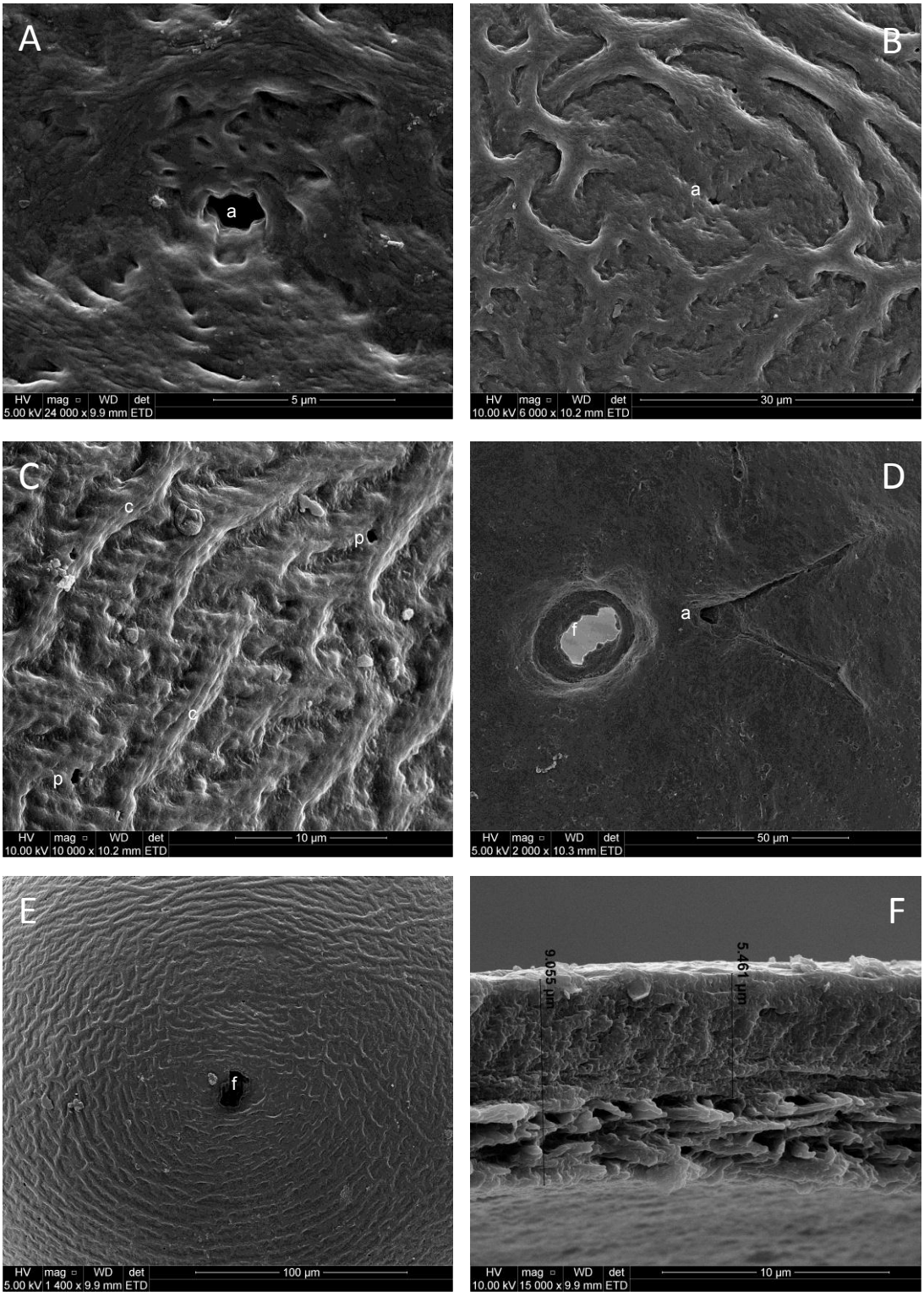

Fig. 7.7. Cysts of population SK18. A: Details of anus; B: Cuticular ornamentation in the vicinity of the anus; C: Punctuations on cyst wall; D: Internal morphology of the cyst wall; E: External morphology of the cyst wall; F: Cross section of cyst wall. For all figures a = anus, c = cuticular ridges, f = vulval fenestra and p = punctuations.

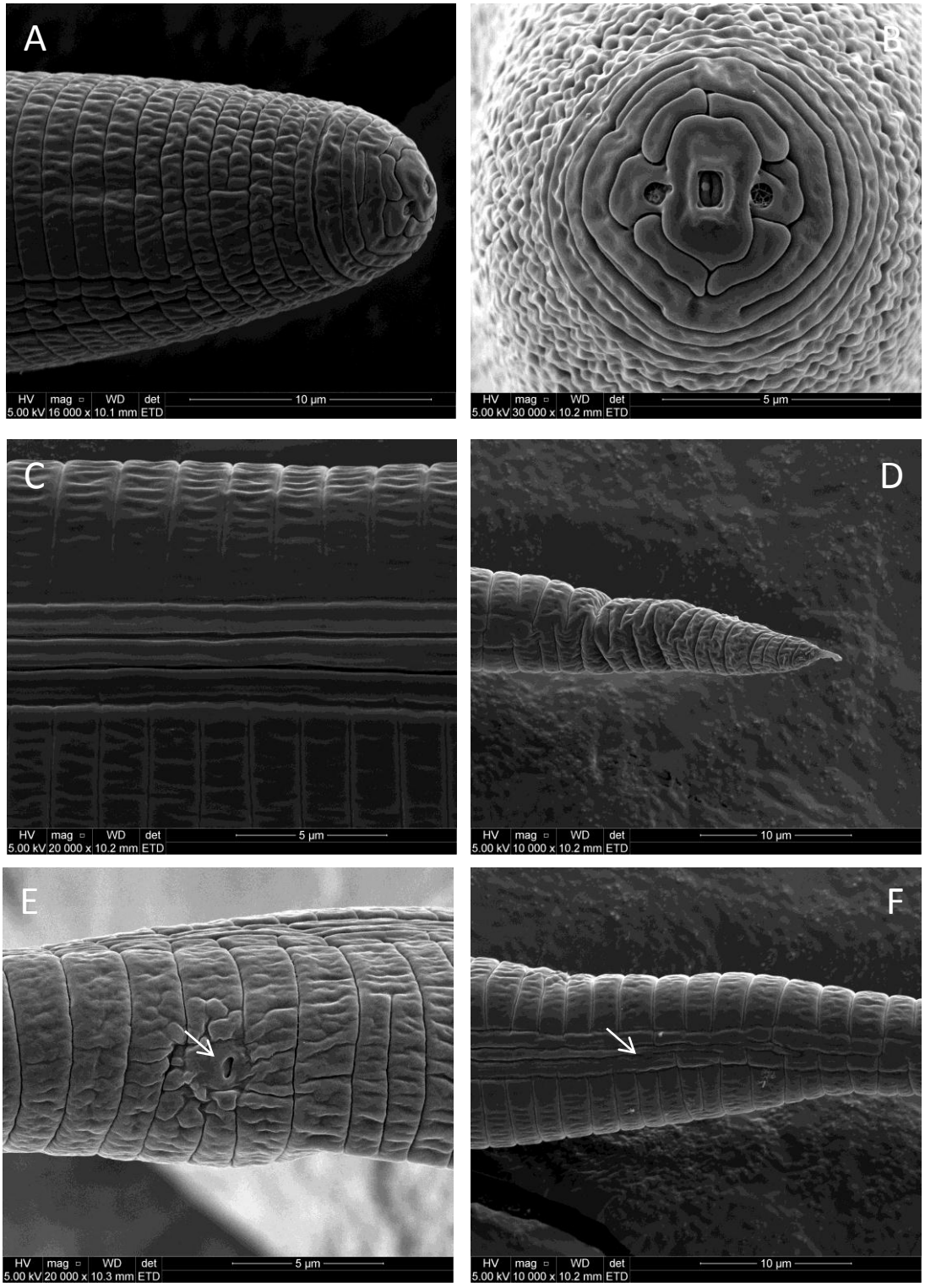

Fig. 7.8. Second stage juveniles (J2) of population SK18 (SEM). **A, B**: Head (A: Lateral view; B: Frontal view); C: Midbody, showing lateral lines; D, E, F: Tail (D: Lateral view of whole tail; E: Ventral view of the tail area, arrow showing the anus F: Lateral view of the ending of lateral lines, arrow showing the phasmid).

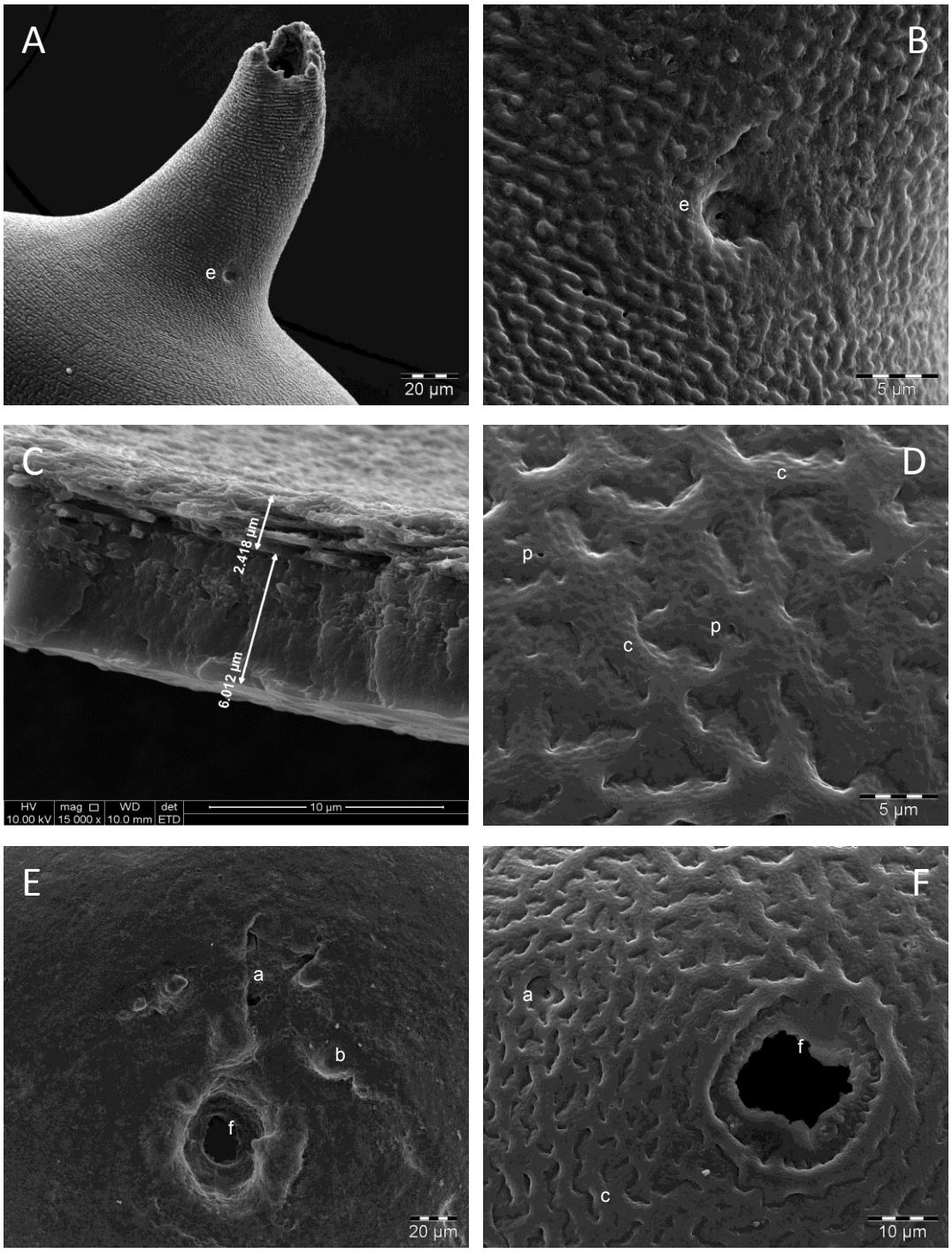

Fig. 7.9. Cysts of population WK1. A: Neck of cyst; B: Cuticular ornamentation in the neck area; C: Cross section of cyst wall; D: Punctuations on cyst wall; E: Internal morphology of the cyst wall; F: External morphology of the cyst wall. For all figures a = anus, c = cuticular ridges, f = vulval fenestra, b = vulval bodies / bullae, e = excretory pore and p = punctuations.

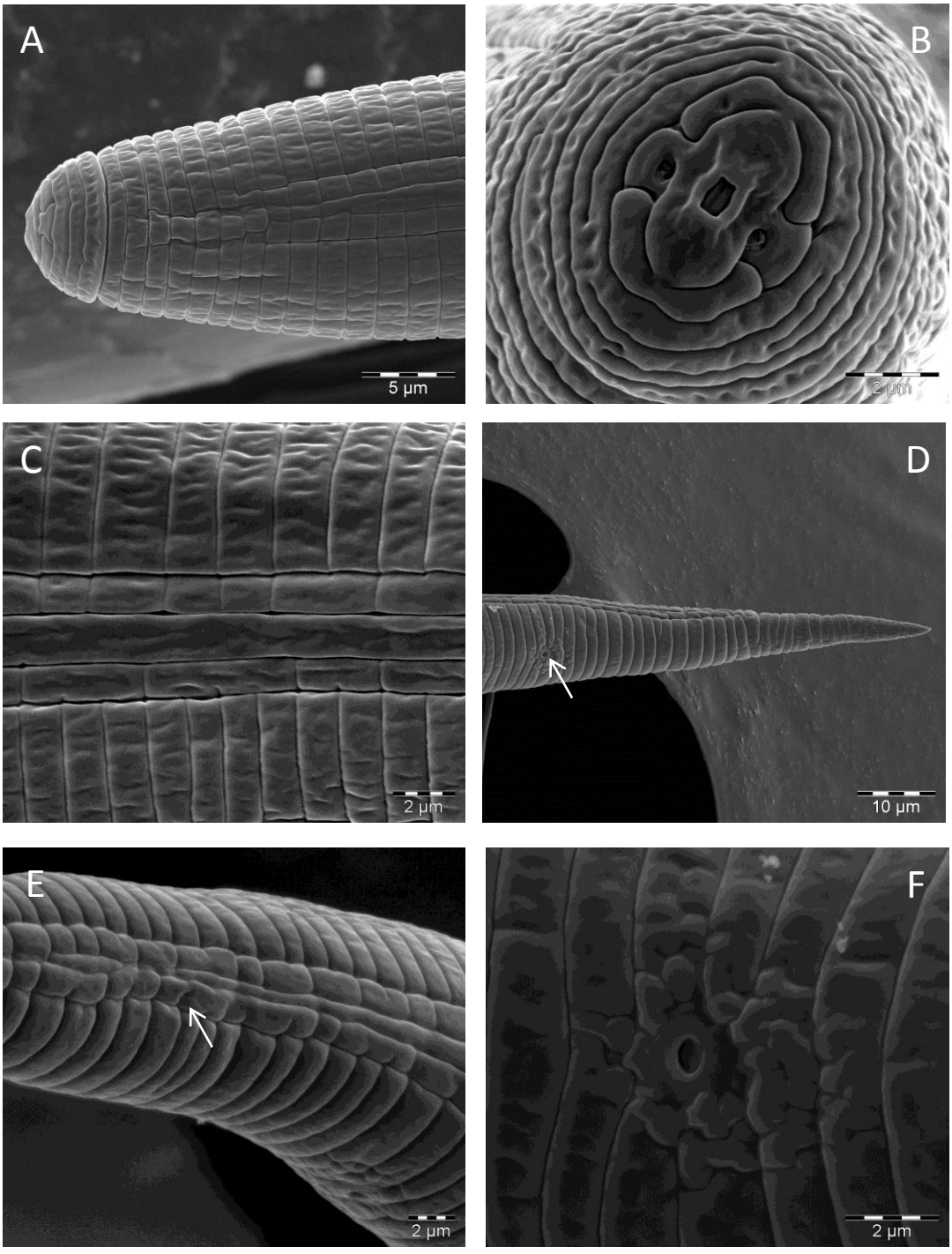

Fig. 7.10. Second stage juveniles (J2) of population WK1 (SEM). A, B: Head (A: Lateral view; B: Frontal view); **C:** Midbody, showing lateral lines; D, E, F: Tail (D: Lateral view of whole tail, arrow showing anus; E: Lateral view of the ending of lateral lines, arrow showing the phasmid, F: Details of the anus, showing ornamentations).

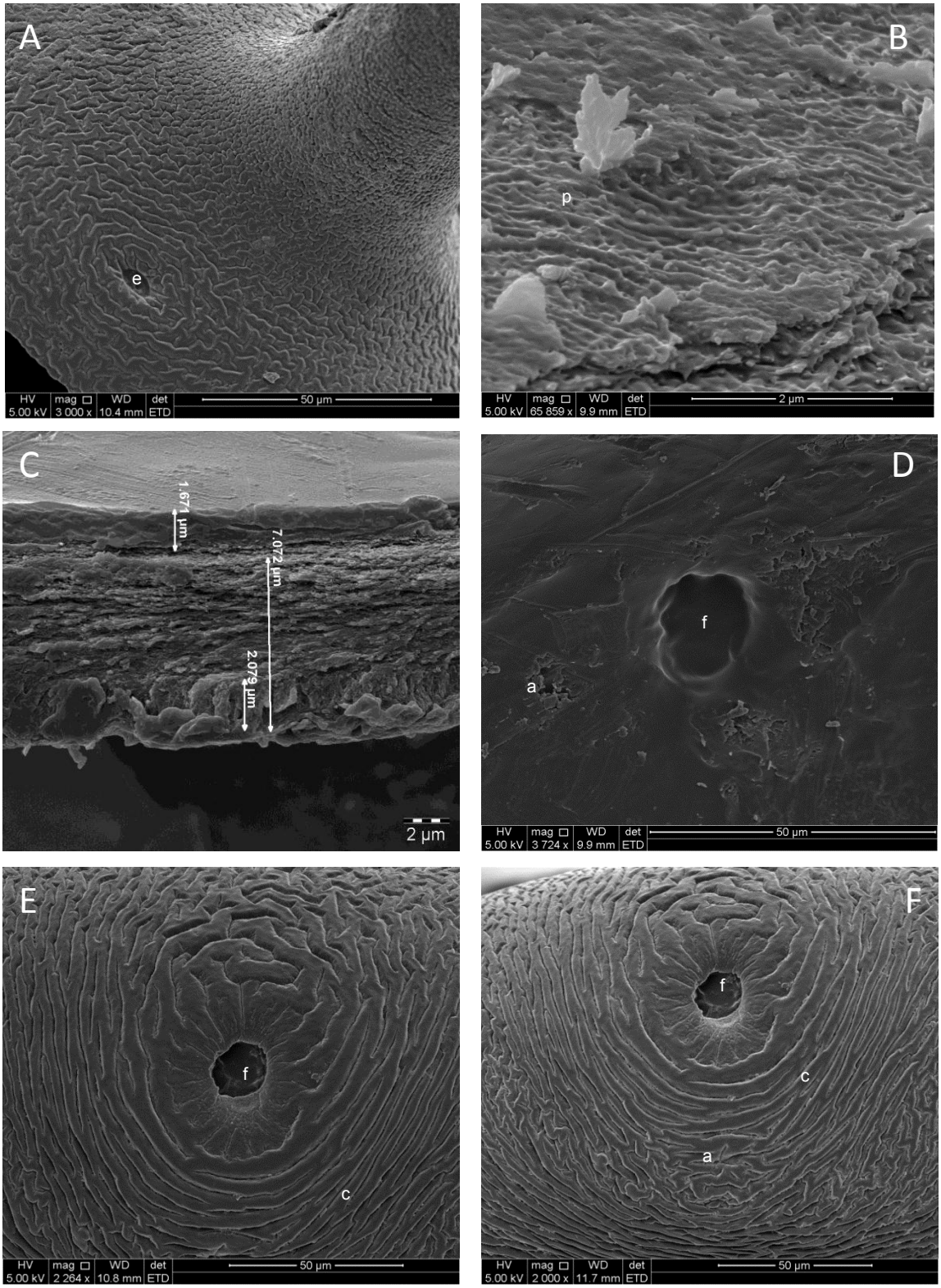

Fig. 7.11. Cysts of population WK2. A: Neck of cyst; B: Ornamentation on cyst wall; C: Cross section of cyst wall; D: Internal morphology of the cyst wall in vulval cone area; E, F: External morphology of the cyst wall in vulval cone area. For all figures a = anus, c = cuticular ridges, f = vulval fenestra, e = excretory pore and p = punctuations.

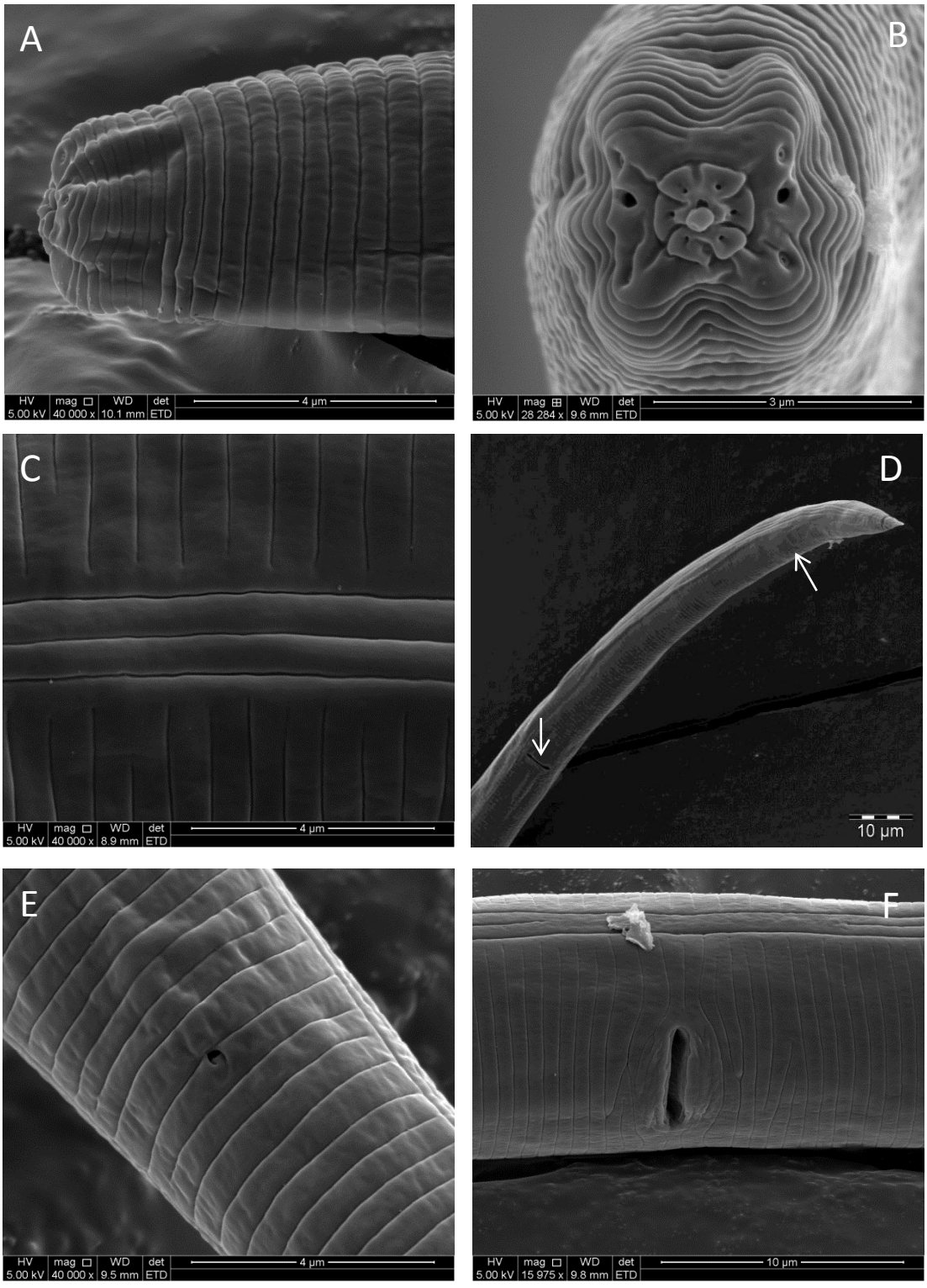

Fig. 7.12. Second stage juveniles (J2) of population WK2 (SEM). A, B: Head (A: Lateral view; B: Frontal view); C: Midbody, showing lateral lines; D: Lateral view of whole tail, arrows showing anus and vulva; E: Posterior region, anus and vulva; Posterior region, showing vulva.

Chapter 8

Description of three new *Globodera* spp. (Nematoda: Heteroderidae) from South Africa

In part extracted from the published manuscript: Knoetze, R. Swart, A & Tiedt, L.R. (2013). Description of *Globodera capensis* n. sp. (Nematoda: Heteroderidae) from South Africa. *Nematology* 15: 233-250.

Introduction

According to Subbotin *et al.* (2010), *Globodera* consists of ten species, namely *G. rostochiensis* (Wollenweber, 1923) Skarbilovich, 1959 (the type species); *G. artemisiae* (Eroshenko & Kazachenko, 1972) Behrens, 1975; *G. bravoae* Franco, Cid del Prado & Lamothe-Argumedo, 2000; *G. leptonepia* (Cobb & Taylor, 1953) Skarbilovich, 1959; *G. mali* (Kirjanova & Borisenko, 1975) Behrens, 1975; *G. mexicana* Subbotin, Mundo-Ocampo & Baldwin, 2010; *G. millefolii* (Kirjanova & Krall, 1965) Behrens, 1975; *G. pallida* Stone, 1973; *G. tabacum* (Lownsbery & Lownsbery, 1954) Behrens, 1975 (with its three subspecies) and *G. zelandica* Wouts, 1984. Potato cyst nematodes (PCN) have been recorded from the African continent from Libya, Algeria, Morocco, Tunisia, Egypt, Sierra Leone, Mozambique and South Africa (Kleynhans, 1998), but no indigenous *Globodera* spp. has so far been found in Africa.

Systematics of the genus *Globodera* Skarbilovich, 1959 (Behrens, 1975)

DIAGNOSIS

(After Siddiqi, 2000 and Subbotin *et al.*, 2010)

Mature female and cyst: Spheroidal with a short projecting neck, terminal region not forming a cone. Cyst brown, surface with a lace-like pattern. Vulva terminal; slit length less than 15 µm long, usually lost from old cysts; tuberculate area near vulva present. Vulval fenestra circumfenestrate. Vaginal remnant underbridge and bullae absent. Anus dorsally subterminal, not on dorsal lip, separated from vulva by a short distance. No anal fenestra. Eggs retained in body; no egg mass formed. *Male*: Cuticle annulated; lateral field with four incisures, outer bands often areolated. Cephalic region offset by constriction, with indistinct labial disc, and three to seven annules. Spicules over 30 µm long, with single pointed tip. Tail short, hemispherical; cloacal lips not forming a tube. *Second-stage juvenile***:** Body slender, straight to arcuate. Stylet less than 30 µm long. *En face* pattern typically with six separate lips, sometimes with fusion of adjacent submedial lips. Tail conically pointed, with hyaline portion about half its total length.

TYPE SPECIES

Globodera rostochiensis (Wollenweber, 1923) Skarbilovich, 1959

syn. *Heterodera schachtii rostochiensis* Wollenweber, 1923

Heterodera rostochiensis Wollenweber, 1923

Heterodera (Globodera) rostochiensis (Wollenweber, 1923) Skarbilovich, 1959

Heterodera schachtii solani Zimmermann, 1927

Heterodera solani Zimmermann, 1927

Heterodera pseudorostochiensis Kirjanova, 1963

Globodera pseudorostochiensis (Kirjanova, 1963) Mulvey & Stone, 1976

Globodera arenaria Chizhov, Udalova & Nasonova, 2008

OTHER SPECIES

G. artemisiae (Eroshenko & Kazachenko, 1972) Behrens, 1975

syn. *Heterodera artemisiae* Eroshenko & Kazachenko, 1972

Globodera hypolysi Ogawa, Ohshima & Ichinohe, 1983

G. bravoae Franco, Cid del Prado & Lamothe-Argumedo, 2000

G. ellingtonae Handoo, Carta, Skantar & Chitwood, 2012

G. leptonepia (Cobb & Taylor, 1953) Skarbilovich, 1959

syn. *Heterodera leptonepia* Cobb & Taylor, 1953

Heterodera (Globodera) leptonepia (Cobb & Taylor, 1953) Skarbilovich, 1959

G. mali (Kirjanova & Borisenko, 1975) Behrens, 1975

syn. *Heterodera mali* Kirjanova & Borisenko, 1975

Globodera mali (Kirjanova & Borisenko, 1975) Mulvey & Stone, 1976

G. mexicana (Campos-Vela, 1967) Subbotin, Mundo-Ocampo & Baldwin, 2010

syn. *Heterodera mexicana* Campos-Vela, 1967

G. millefolii (Kirjanova & Krall, 1965) Behrens, 1975

syn. *Heterodera millefolii* Kirjanova & Krall, 1965

Heterodera (Globodera) millefolii (Kirjanova & Krall, 1965) Mulvey, 1973

Globodera achilleae (Golden & Klindic, 1973) Behrens, 1975

Heterodera achilleae Golden & Klindic, 1973

G. pallida Stone, 1973a

syn. *Heterodera pallida* Stone, 1973a

Heterodera (Globodera) pallida Stone, 1973a

G. tabacum tabacum (Lownsbery & Lownsbery, 1954) Skarbilovich, 1959

syn. *Heterodera tabacum* Lownsbery & Lownsbery, 1954

Globodera tabacum (Lownsbery & Lownsbery, 1954) Behrens, 1975

G. tabacum solanacearum (Miller & Gray, 1972) Behrens, 1975

syn. *Heterodera solanacearum* Miller & Gray, 1972

Heterodera tabacum solanacearum (Miller & Gray, 1972) Stone, 1983

Globodera solanacearum (Miller & Gray, 1972) Behrens, 1975

G. tabacum virginiae (Miller & Gray, 1968) Stone, 1973

syn. *Heterodera virginiae* Miller & Gray, 1968

Heterodera tabacum virginiae (Miller & Gray, 1968) Stone, 1983

Globodera virginiae (Miller & Gray, 1968) Behrens, 1975

G. zelandica Wouts, 1984

During the systematic sampling of potato-producing areas of South Africa (Knoetze *et al.*, 2006), a new cyst nematode was found on several farms in the Swartland and Sandveld areas. The discovery of one new *Globodera* species in South Africa, suggesting the possibility of the discovery of more new representatives of *Globodera* from Southern Africa, prompted a survey of the Cape Floristic Region (CFR) for the presence of indigenous cyst nematodes. The survey (Chapter 2), unearthed a further four possible new species of cyst nematodes. Morphological and molecular studies (Chapters 4 and 7) indicated that three of these discoveries can be regarded as new species of *Globodera.*

Materials and methods

MORPHOLOGICAL AND MORPHOMETRIC STUDY

For light microscopy and scanning electron microscopy techniques, refer to Chapter7.

MOLECULAR STUDY

For molecular and phylogenetic techniques, refer to Chapter 4.

Results

The host plant of these *Globodera* spp. is, to date, unknown and consequently the species is represented only by the second-stage juveniles (J2) and young and mature cysts that were extracted from soil. The morphological descriptions and molecular characterisations of three species, *Globodera capensis* n. sp., *Globodera* sp. SK18 and *Globodera* sp. WK1 are presented in this section.

***Globodera capensis*[1] n. sp.**

(Figs. 8.1, 7.2, 7.3, 7.4, 7.5, 7.6)

MEASUREMENTS

See Tables 7.6, 7.7

DESCRIPTION

Cysts

Cysts small to large, yellow to dark brown in colour, varying from turbiniform, subglobose to spherical with short neck. Perineal region circumfenestrate with small, subterminal anus at apex of V-shaped subsurface mark in cuticle of some specimens. Vulva a straight transverse slit lying between papillated crescentic areas. Bullae or vulval bodies present in majority of cysts, varying from slight thickenings of inner surface cuticle around vulval basin to large, variously shaped knobs. Bullae may occupy extensive areas around anus and vulval basin, may form a ring around vulval basin or be confined to a few small, isolated knobs around vulval basin. Six to eleven parallel cuticular ridges on outer surface of cyst between anus and vulval basin, sometimes very faint and almost invisible with LM; these ridges changing to

[1]The species is named after the locality where it was found, namely, the Western Cape, South Africa. The description of this species has already been published as part of this study (Knoetze *et al.*, 2013). This species is also called SP25 in the rest of the manuscript.

irregular patterns in area beyond vulval basin and around and just beyond anus, and again to crescentic wavy ridges, extending to neck-area. Irregular subsurface dots generally visible over whole body, appearing to be arranged in parallel rows at right angle to the long axis of some cysts.

Second stage juvenile (J2)

Body curved slightly ventrally. Tail tapering to finely rounded terminus. Head with three annules and medial lips with labial disc, set off from rest of body. With SEM, medial lips and labial disc oval-rectangular in face view, raised above lateral lips. Prestoma opening rectangular slightly raised above rest of labial disc and medial lips. Lateral lips large, rounded, bearing amphid apertures; separating first labial annule to form two medial crescents. Stylet long and thin with cone occupying less than 50 % of total stylet length. Stylet knobs rounded to flattened; DGO (dorsal gland outlet to stylet knobs), 4 – 7 µm long. Oesophageal gland lobe extending to about 30 % of total body length. Gonadial primordium situated at 50 – 58 % of body length. Nerve ring situated short distance posterior to median bulb with excretory pore at 1 – 1.5 body widths posterior of nerve ring. Hemizonid, hemizonion and cephalids not seen. Annules about 1.8 µm wide at midbody. Lateral fields with four incisures, extending from about six annules posterior of labial region to 15 – 20 µm from tail tip (SEM), incisures crenated, areolated. Phasmids on average situated slightly more than one anal body width posterior to anus, indistinct in many specimens. Tail terminus annulated to tip (SEM), varies from relatively smooth to irregular in appearance (LM).

Eggs (n = 10)

Length = 90 ± 7.2 (77.7-99.9) µm; diam. = 39.4 ± 4.44 (33.3-44) µm; L/D ratio = 2.3. No visible markings on egg shell.

TYPE HABITAT AND LOCALITY

Potato field on Rhenosterfontein farm near Porterville in the Fynbos Biome of the Western Cape Province, South Africa (32º51.413'S, 18º55.862'E) on 4 April 2001. It was subsequently found in the rhizosphere of *Conicosia pugioniformis* (pig's root), which are commonly seen as opportunistic plants on potato fields.

TYPE MATERIAL

Holotype cyst (female) on slide 40543 and 9 paratype cysts and 8 paratype J2 on slides 40537-40559 deposited in the National Collection of Nematodes, ARC-PPRI, Pretoria, South Africa. Two paratype cysts and eight paratype J2 deposited at the National Collection of New Zealand (NNCZN), Tamaki Campus, University of Auckland, New Zealand.

DIAGNOSIS AND RELATIONSHIPS

Cysts of *G. capensis* n.sp. are characterized by their turbiniform, subglobose to spherical shapes, short neck, the presence of subcuticular punctations over the whole body and bullae in the terminal area of most specimens. Six to 20 cuticular ridges/lines are present on the

outer surface of the cyst between the anus and vulval basin. Granek's ratio ranges from 0.7 - 4.0, the vulval basin diameter from 16 - 28 µm and the distance between vulval basin and anus from 19 – 60 µm. The J2 is characterized by a long thin stylet of 23 – 28 µm with rounded to anteriorly flattened knobs. The dorsal oesophageal gland outlet to the stylet knobs is 4 – 7 µm long. The tail is 44 – 73 µm long and the length of the hyaline terminal is 24 – 41.5 µm or 42 – 80 % of the tail length

Following the key to the species of *Globodera* in Subbotin *et al.* (2010), *G. capensis* n. sp. is morphologically and morphometrically near or indistinguishable from *G. tabacum, G. artemisiae* and *G. millefolii*. As the species is also near PCN and it has been found in potato fields, *G. capensis* will also be morphologically and morphometrically compared with *G. rostochiensis* and *G. pallida*. The morphometric and morphological data used in the relationships between *G. capensis* n. sp. and comparable species were taken from Fleming & Powers (1998) and Stone (1973b, c) unless stated otherwise. According to Fleming & Powers (1998) and Evans & Rowe (1998), *G. tabacum* consists at present of three subspecies, *G. tabacum solanacearum* (Miller & Gray, 1972) Behrens, 1975, *G. tabacum tabacum* (Lownsbery & Lownsbery, 1954) Behrens, 1975 and *G. tabacum virginiae* (Lownsbery & Lownsbery, 1954) Behrens, 1975. In the non-maze-like pattern of cuticular ridges between the vulval basin and anus, *G. capensis* n. sp. is more similar to *G. tabacum tabacum* (maze-like in *G. tabacum solanacearum* and *G. tabacum virginiae*). In Granek's ratio however, *G. capensis* n. sp. is closer to all three subspecies: G = 0.7-4.0 vs 0.9-2.8 for *G. tabacum tabacum*, 1.5-4.2 for *G. tabacum virginiae* and 1.3-3.8 for *G. tabacum solanacearum*. Likewise, in its turbiniform, subglobose to spherical cyst shape, *G. capensis* n. sp. is similar to all three subspecies, viz., turbiniform to subglobose in *G. tabacum solanacearum* and *G. tabacum virginiae,* and spherical to globose in *G. tabacum tabacum*. *Globodera capensis* n.

sp. can be differentiated from *G. rostochiensis* by the presence of prominent bullae in the terminal region of most specimens vs abullate but with small vulval bodies in some specimens of *G. rostochiensis*, lower Granek's ratio of 1.3-9.5 vs >3, lower number of cuticular ridges between vulval basin and anus (6-20 vs 12-31 with the mean > 14), shorter distance from vulval basin to anus of 19-60 vs 37-77 µm with the mean > 55) and larger vulval basin diameter of 16.0-28.5 vs 8-20 µm with the mean < 19). *G. capensis* n. sp. differs from *G. pallida* mainly by smaller cysts (mean length and width: 447 × 390 vs 579 × 534 µm), presence of prominent bullae in the terminal region of most specimens vs abullate but with small vulval bodies in some specimens, and larger vulval basin diam. of 18-21 vs >19. *Globodera capensis* n. sp. differs from *G. artemisiae* mainly by the higher Granek's ratio and greater distance between anus and vulval basin (2.0 vs 1.0 and 37.5 vs 26.0 µm, respectively). The morphology and morphometrics of the cysts of *G. artemisiae* were taken from Eroshenko & Kazachenko (1972). *Globodera capensis* n. sp. can be distinguished from *G. millefolii* by the short vulval slit of 10-11 vs ca 35 µm and the smaller and rounder eggs of 90 (77.7-99.9) × 39 (33.3-44.0) vs 132 (122-140) × 49 (45.0-52.0) µm; L/W ratio=2.3 vs 2.69. The morphometrics and morphology of the cysts and eggs of *G. millefolii* were taken from Kirjanova & Krall (1971) and Sturhan (1984). The J2 of *G. capensis* n. sp. can be distinguished from *G. tabacum* only by the lower number of labial annules (3 vs 4-6). It can be distinguished from both species of PCN mainly by the longer average stylet (24.1-26.3 vs 23.8 µm in *G. pallida* and 22 µm in *G. rostochiensis*) and higher mean DGO (4.2-5.4 µm in *G. capensis* n. sp. vs 2.7 µm in *G. pallida* and 2.6 µm in *G. rostochiensis*). In *G. capensis* n. sp. the shape of the medial lips and labial disc is comparable with that of *G. rostochiensis* (oval-rectangular), but differs from that of *G. pallida* (rectangular medial lips and labial disc). The J2 of *G. capensis* n. sp. differs from that of *G. artemisiae* mainly in the longer body (L = 430-528 vs 355-419 µm), but also in the longer mean tail and stylet lengths of 47.1-54.6 vs

40.0 µm and 24.1-26.3 vs 22.6 µm, respectively (the morphology and morphometrics of the J2 of *G.artemisiae* were taken from Eroshenko & Kazachenko,1972). *Globodera capensis* n. sp. can be differentiated from *G. millefolii* by the longer stylet of 24.1-26.3 vs 22 µm and three vs five head annules (morphometrics of J2 *G. millefolii* were taken from Sturhan, 1984).

MOLECULAR CHARACTERISATION

Polymerase chain reaction

Amplification of the ITS regions of *G. capensis* n. sp. with primers rDNA1 and rDNA2 yielded a single fragment of 1210 bp.

DNA sequence characterisation

ITS-rDNA sequences from *G. capensis* n. sp. were submitted to the GenBank database under the accession numbers JQ595409-JQ595413. The sequence of ITS regions of *G. capensis* n. sp., including ITS1 + 5.8S + ITS2 can be recognised by its length of 917 base pairs (ITS1 = 543 bp; ITS2 = 203 bp). Sequence lengths and frequencies of nucleotide distribution for *G. capensis* n. sp., as well as closely related species, are shown in Table 4.2.

Pairwise distances between closely related *Globodera* species (Table 4.3) show that *G. capensis* n. sp differs least from *G. millefolii* ((base differences per sequence = 60; number of nucleotide substitutions per site = 0.070), and most from *G. ellingtonae* and an undescribed *Globodera* from Chile (base differences per sequence = 70; number of nucleotide substitutions per site = 0.083).

Phylogenetic relationships

Phylogenetic relationships of *G. capensis* n. sp. with selected species of the Heteroderidae inferred from ITS-rRNA sequences by using the Maximum Parsimony method (MP) are given in Chapter 4 (Fig. 4.3). The consensus tree consisted of seven major clades with adequate bootstrap support (BS). They are: *i) Globodera* parasitizing non-solanaceous plants (BS = 86); *ii) Globodera* parasitizing solanaceous plants (BS = 100); *iii) Paradolichodera; iv) Punctodera* (BS = 100)*; v) Cactodera* (BS = 100), *Heterodera* (BS = 96) and *vii) Betulodera*. The position of *G. capensis* n. sp. in our tree corresponds with the findings of Subbotin *et al*. (2011), occupying a basal position within a lineage of *Globodera* species from Europe, Asia and New Zealand, parasitizing non-Solanaceous plants.

Globodera sp. SK18

(Fig. 8.2, 7.2, 7.3, 7.4, 7.7, 7.8)

MEASUREMENTS

See Tables 7.6, 7.7

DESCRIPTION

Cysts

Cysts, small to medium in size, light brown to dark brown in colour, ovate to spherical in shape with short neck. Perineal region circumfenestrate with small, subterminal anus at apex of V-shaped subsurface mark in cuticle of some specimens. Bullae or vulval bodies absent. Six to twelve parallel cuticular ridges on outer surface of cyst between anus and vulval basin, clearly visible with LM; these ridges changing to irregular patterns in area beyond vulval basin and around and just beyond anus, and again to crescentic wavy ridges, extending to neck-area. Punctations generally present but variable in intensity and arrangement. Irregular subsurface dots generally visible over whole body, appearing to be arranged in parallel rows at right angle to the long axis of some cysts.

Second stage juvenile (J2)

Body curved slightly ventrally. Tail tapering to finely pointed terminus. Head with three to four annules and medial lips with labial disc, set off from rest of body. With SEM, the medial

lips and labial disc are oval-rectangular in face view, not raised above lateral lips. Prestoma opening rectangular, slightly raised above rest of labial disc and medial lips. Lateral lips large, rectangular, sometimes irregular in shape, bearing amphid apertures. Stylet well developed. Stylet knobs rounded to flattened. DGO (dorsal gland outlet to stylet knobs), 3.5-6.5 µm long. Oesophageal gland lobe extending to about 35 % of total body length. Genital primordium slightly posterior to mid-body. Nerve ring situated short distance posterior to median bulb with excretory pore at less than 1 body width posterior of nerve ring. Valve of median bulb prominent. Hemizonid, hemizonion and cephalids not seen. Annules about 1.7 µm wide at midbody. Lateral fields with four incisures, extending from about six annules posterior of labial region to 15 – 20 µm from tail tip (SEM), incisures crenated, areolated. Phasmid indistinct in most specimens. Tail terminus annulated to tip (SEM), relatively smooth in appearance (LM).

Eggs (n = 11)

Length = 107.6 ± 5.1 (95.6-113.2) µm; diam. = 44.0 ± 2.5 (40.6-49.8) µm; L/D ratio = 2.5 ± 0.2 (2.1-2.7). No visible markings on egg shell.

TYPE HABITAT AND LOCALITY

Disturbed veldt in Agulhas Limestone Fynbos, in an area dominated by the plant genera *Solanum* and *Chrysanthemoides*, near the town of Gansbaai in the Fynbos Biome of the Western Cape Province, South Africa (34° 33.328'S; 19° 26.195'E) on 20 July 2010.

TYPE MATERIAL

Holotype cyst (female) on slide 40564, 3 paratype cysts on slides 40565, 40566 and 40567 and 6 paratype J2 on slides 40568 and 40569 deposited in the National Collection of Nematodes, ARC-PPRI, Pretoria, South Africa.

DIAGNOSIS AND RELATIONSHIPS

Cysts of *Globodera* sp. (SK18) are characterized by their ovate to spherical shape, short neck, the presence of subcuticular punctations over the whole body and the absence of bullae or vulval bodies. Six to 12 cuticular ridges/lines are present on the outer surface of the cyst between the anus and vulval basin. Granek's ratio ranges from 1.0 - 3.0, the vulval basin diameter from 11.7 - 26.1 µm and the distance between vulval basin and anus from 19.1 - 47.0 µm. The J2 is characterized by a well developed stylet of 22.5 - 24.8 µm with rounded to anteriorly flattened knobs. The dorsal oesophageal gland outlet to the stylet knobs is 3.5 - 6.5 µm long. The tail is 48.8 - 63.9 µm long and the length of the hyaline terminal is 18.7 - 29.3 µm long.

Following the key to the species of *Globodera* in Subbotin *et al.* (2010), these specimens are morphologically and morphometrically near or indistinguishable from *G. artemisiae and G. millefolii*. The cysts from *Globodera* sp. SK18 are slightly smaller and rounder than the cysts of *G. artemisiae* as described by Eroshenko & Kazachenko (1972) (mean length and width: 417 × 379 vs 493 × 356 µm). The vulval basin diameter of these cysts are less than that of *G. artemisiae* as described by Eroshenko & Kazachenko (1972) (17.6 vs 24.9), resulting in a

higher Granek's ratio (1.7 vs 1.0). These cysts also display a lower number of cuticular ridges between vulval basin and anus (6-12 vs 5-16) as published for *G. artemisiae* by Manduric & Andersson (2004). The cysts from *Globodera* sp. SK18 are also smaller than the cysts of *G. millefolii* as described by Golden & Klindić (1973) (mean length and width: 417 × 379 vs 639 × 479 µm). These cysts also differ from *G. millefolii* in having more cuticular ridges between the vulval basin and anus (6-12 vs 4-7) as published for *G. millefolii* by Sirca & Urek (2004). The eggs are slightly longer and narrower than that of *G. artemisiae* as described by Eroshenko & Kazachenko (1972) (107.6 × 44.0 vs 98.7 × 44.7) and smaller than those of *G. millefolii* as described by Golden & Klindić (1973) (108 × 44 vs 110 × 50). The J2 differs from that of *G. artemisiae* in the longer body (L = 416 – 524 vs 357- 490 µm), longer mean tail of 55.7 vs 40.0 µm and three to four vs five to six head annules (the morphology and morphometrics of the J2 of *G.artemisiae* were taken from Eroshenko & Kazachenko,1972 and Subbotin *et al.*, 2010). The J2 of *Globodera* sp. SK18 can be differentiated from *G. millefolii* by the shorter stylet of 22.5-24.8 vs 24-25 µm, shorter distance between dorsal oesophageal gland outlet and stylet knobs (4.4 vs 5.7) and three to four vs five head annules (morphology and morphometrics of J2 of *G. millefolii* were taken from Golden & Klindić, 1973 and Subbotin *et al.*, 2010).

MOLECULAR CHARACTERISATION

Polymerase chain reaction

Amplification of the ITS regions of *Globodera* sp. SK 18 with primers rDNA1 and rDNA2 yielded a single fragment of 1213 bp.

DNA sequence characterisation

The ITS-rDNA sequence from *Globodera* sp. SK18 was submitted to the GenBank database under the accession number KC148543. The sequence of ITS regions of *G. agulhasensis* n. sp., including ITS1 + 5.8S + ITS2 can be recognised by its length of 920 base pairs (ITS1 = 542 bp; ITS2 = 207 bp). Sequence lengths and frequencies of nucleotide distribution for *Globodera* sp. SK18 and closely related species, are shown in Table 4.2.

Pairwise distances between closely related *Globodera* species (Table 4.3) show that *G. agulhasensis* n. sp differs least from *G. millefolii* ((base differences per sequence = 33; number of nucleotide substitutions per site = 0.037), and most from an undescribed *Globodera* from Chile (base differences per sequence = 79; number of nucleotide substitutions per site = 0.095).

Phylogenetic relationships

Phylogenetic relationships of *Globodera* sp. SK18 with selected species of the Heteroderidae inferred from ITS-rRNA sequences by using the Maximum Parsimony method (MP) are given in Chapter 4 (Fig. 4.3). The consensus tree consisted of seven major clades with adequate bootstrap support (BS). They are: *i) Globodera* parasitizing non-solanaceous plants (BS = 86); *ii) Globodera* parasitizing solanaceous plants (BS = 100); *iii) Paradolichodera; iv) Punctodera* (BS = 100)*; v) Cactodera* (BS = 100), *Heterodera* (BS = 96) and *vii) Betulodera*. *Globodera* sp. SK18 is included in the clade of *Globodera* sp. that parasitise non-solanaceous plants, forming a monophyletic group with unidentified *Globodera* spp. from

Portugal, *G. millefolii* and *G. artemisiae* (BS = 99). However when Neighbor-Joining (NJ), Maximum Likelihood (ML) and Maximum Parsimony method (MP) trees are compared (Chapter 4), the position of *Globodera* sp. SK18 in that clade is not clear.

Globodera sp. WK1

(Fig. 8.3, 7.2, 7.3, 7.4, 7.9, 7.10)

MEASUREMENTS

See Tables 7.6, 7.7

DESCRIPTION

Cysts

Cysts medium to large, light brown to dark brown in colour, ovate to spherical in shape with short neck. Perineal region circumfenestrate with small, subterminal anus at apex of V-shaped subsurface mark in cuticle of some specimens. Bullae or vulval bodies present in majority of cysts, varying from slight thickenings of inner surface cuticle around vulval basin to large, variously shaped knobs. Bullae may occupy extensive areas around anus and vulval basin, may form a ring around vulval basin or be confined to a few small, isolated knobs around vulval basin. Eight to fourteen parallel cuticular ridges on outer surface of cyst between anus and vulval basin, sometimes very faint and almost invisible with LM; these ridges changing to irregular patterns in area beyond vulval basin and around and just beyond anus, and again to crescentic wavy ridges, extending to neck-area. Punctations generally present but variable in intensity and arrangement. Irregular subsurface dots generally visible over whole body, appearing to be arranged in parallel rows at right angle to the long axis of some cysts.

Second stage juvenile (J2)

Body curved slightly ventrally. Tail tapering to finely rounded terminus. Head with three annules and medial lips with labial disc, set off from rest of body. With SEM, medial lips and labial disc oval-rectangular in face view, raised above lateral lips. Adjacent submedial lips are fused with one another and with the labial disc in some specimens. Prestoma opening rectangular, slightly raised above rest of labial disc and medial lips. Lateral lips rectangular, bearing amphid apertures. Stylet long and well developed with cone occupying almost 50 % of total stylet length. Stylet knobs rounded to flattened; DGO, 4.5 – 7.5 µm long. Oesophageal gland lobe extending to about 30 % of total body length. Genital primordium slightly posterior to mid-body. Nerve ring indistinct in most specimens. Valve of median bulb prominent. Hemizonid, hemizonion and cephalids not seen. Annules about 1.7 µm wide at midbody. Lateral fields with four incisures, extending from about six annules posterior of labial region to about 20 µm from tail tip (SEM), incisures crenated, areolated. Incusers disrupted around anus to form anal ornamentations (SEM). Phasmid indistinct in most specimens. Tail terminus annulated to tip (SEM), varies from relatively smooth to irregular in appearance (LM).

Eggs (n = 11)

Length = 134.3 ± 8.8 (116.6-147.6) µm; diam. = 49.2 ± 2.8 (44.0-54.2) µm; L/D ratio = 2.7 ± 0.3 (2.3-3.1). No visible markings on egg shell.

TYPE HABITAT AND LOCALITY

Natural veldt in Leipoldtville Sand Fynbos, in an area dominated by the plant genera *Indogifera, Wiborgia* and *Lampranthus*, near the town of Leipoldtville in the Sandveld region of the Western Cape Province, South Africa (32° 13.400' S; 18° 28.703' E) during May 2010.

TYPE MATERIAL

Holotype cyst (female) on slide 40570, 3 paratype cysts on slides 40571, 40572 and 40573 and 7 paratype J2 on slides 40574 and 40575 deposited in the National Collection of Nematodes, ARC-PPRI, Pretoria, South Africa.

DIAGNOSIS AND RELATIONSHIPS

The cysts of *Globodera* sp. WK1 are characterized by their ovate to spherical shape, short neck, the presence of subcuticular punctations over the whole body and bullae in the terminal area of most specimens. Eight to 14 cuticular ridges/lines are present on the outer surface of the cyst between the anus and vulval basin. Granek's ratio ranges from 1.0 – 2.5, the vulval basin diameter from 15.6 - 25.0 µm and the distance between vulval basin and anus from 19.9-53.5 µm. The J2 is characterized by a long and well developed stylet of 25.3-28.5 µm with rounded to anteriorly flattened knobs. The dorsal oesophageal gland outlet to the stylet knobs is 4.6-7.2 µm long. The tail is 57.3-76.8 µm long and the length of the hyaline terminal is 21.6 - 38.8 µm long.

Following the key to the species of *Globodera* in Subbotin *et al.* (2010), these specimens are morphologically and morphometrically near to *G. millefolii, G. zelandica and G. bravoae*. These cysts can be differentiated from *G. millefolii, G. zelandica and G. bravoae* by the presence of prominent bullae in the terminal region of most specimens vs abullate for *G. millefolii, G. zelandica and G. bravoae* (Subbotin *et al.* 2010). The cysts from *Globodera* sp. WK1 are slightly smaller than the cysts of *G. bravoae* as described by Franco *et al.* (2000) (mean length and width: 549 × 486 vs 739 including neck × 579 µm). The mean vulval basin diameter of these cysts are less than that of *G. bravoae* as described by Franco *et al.* (2000) (21.0 vs 27.0) and so is the distance from vulval basin to anus (33.6 vs 47.5), resulting in a slightly lower Granek's ratio (1.6 vs 1.8). The cysts from SK18 are shorter in length than the cysts of *G. millefolii* as described by Golden & Klindić (1973) (549 vs 639). The vulval basin diameter and distance from vulval basin to anus of these cysts are more than that of *G. millefolii* as described by Golden & Klindić (1973) (21.0 vs 16.0 and 33.6 vs 27, respectively). These cysts also differ from *G. millefolii* in having more cuticular ridges between the vulval basin and anus (8-14 vs 4-7) as published for *G. millefolii* by Sirca & Urek (2004). The eggs are larger than those of *G. bravoae* as described by Franco *et al.* (2000) (134.3 × 49.2 vs 113.5 × 44.2) and *G. millefolii* as described by Golden & Klindić (1973) (134.3 × 49.2 vs 110 × 50). The J2 of *Globodera* sp. WK1 differs from that of *G. bravoae* in the longer body (L = 545 - 630 vs 442 - 553 µm), longer stylet length (25.3 - 28.5 vs 18.4 – 26.8 µm) shorter mean DGO (5.6 vs 6.8 µm) and longer mean tail of 63.9 vs 56.2 µm (the morphology and morphometrics of the J2 of *G. bravoae* were taken from Franco *et al.*, 2000 and Subbotin *et al.*, 2010). The J2 of *G.* n. sp. can be differentiated from *G. millefolii* by the longer body length (L = 545 - 630 vs 472 - 515 µm), longer stylet of 25.3 - 28.5 vs 24.0 – 25.0 µm, longer mean tail of 63.9 vs 55.0 µm, longer tail hyaline region of 21.6 - 38.8 vs 20.0 – 32.0 µm and three vs five head annules (morphology and morphometrics

of J2 of *G. millefolii* were taken from Golden & Klindić, 1973 and Subbotin *et al.*, 2010). The J2 of *Globodera* sp. WK1 can be differentiated from *G. zelandica* by the longer body length (L = 545 - 630 vs 429 - 511 µm), shorter stylet (25.3 - 28.5 vs 26.5 – 30.0 µm), longer mean DGO (5.6 vs 4.5 µm) shorter tail hyaline region (21.6 - 38.8 vs 29.0 – 44.0 µm) and three vs four head annules (morphology and morphometrics of J2 of *G. zelandica* were taken from Wouts, 1984 and Subbotin *et al.*, 2010).

MOLECULAR CHARACTERISATION

Polymerase chain reaction

Amplification of the ITS regions of *Globodera* sp. WK1 with primers rDNA1 and rDNA2 yielded a single fragment of 1205 bp.

DNA sequence characterisation

The ITS-rDNA sequence from *Globodera* sp. WK1 was submitted to the GenBank database under the accession number KF809767. The sequence of ITS regions of *Globodera* sp. WK1, including ITS1 + 5.8S + ITS2 can be recognised by its length of 913 base pairs (ITS1 = 538 bp; ITS2 = 204 bp). Sequence lengths and frequencies of nucleotide distribution for *Globodera* sp. WK1 and closely related species, are shown in Table 4.2.

Pairwise distances between closely related *Globodera* species (Table 4.3) show that *Globodera* sp. WK1 differs least from *G. millefolii* ((base differences per sequence = 39;

number of nucleotide substitutions per site = 0.045), and most from *G. mexicana* (base differences per sequence = 77; number of nucleotide substitutions per site = 0.092).

Phylogenetic relationships

Phylogenetic relationships of *Globodera* sp. WK1 with selected species of the Heteroderidae inferred from ITS-rRNA sequences by using the Maximum Parsimony method (MP) are given in Chapter 4 (Fig. 4.3). The consensus tree consisted of seven major clades with adequate bootstrap support (BS). They are: *i) Globodera* parasitizing non-solanaceous plants (BS = 86); *ii) Globodera* parasitizing solanaceous plants (BS = 100); *iii) Paradolichodera; iv) Punctodera* (BS = 100)*; v) Cactodera* (BS = 100), *Heterodera* (BS = 96) and *vii) Betulodera*. *Globodera* sp. WK1 is included in the clade of *Globodera* sp. that parasitise non-solanaceous plants, occupying a basal position in a monophyletic group containing unidentified *Globodera* spp. from Portugal, *G. millefolii* and *G. artemisiae* (BS = 99).

Discussion

Subbotin *et al.* (2011) speculated that South America or Africa appears to be a centre of origin of *Globodera*, supporting Stone's (1979) hypothesis of a Gondwanaland origin of *Globodera* with subsequent dispersal of the species of this genus to Europe, North America, Asia and Oceania. Subbotin *et al.* (2011) suggested that the divergence of the two main *Globodera* lineages might have occurred subsequent to the break-up of Africa and South America in the Mid-Cretaceous, because of the association thereof with the time of origin for the Solanaceae. Subbotin *et al.* (2011) also argued that the evolution of the Punctoderinae

cannot be explained solely by the separation of the continents and diffusion expansion, suggesting a scenario whereby the ancestral Punctoderinae gave rise to a modern *Globodera* lineage that was introduced to South America or Africa via long distance dispersal from North America. The discovery of these new *Globodera* species in Southern Africa supports the theory of a Gondwanaland origin of the genus and the theory that divergence of the two main *Globodera* lineages might have occurred subsequent to the break-up of Africa and South America. The ancestors of the *Globodera* species parasitizing the Asteraceae in Europe were suggested to have been carried northwards when fragments of Gondwanaland encountered Laurasia creating an isolated evolutionary niche, where these species might have co-evolved with their hosts (Stone, 1983).

Although the host plants of the South African *Globodera* species have not yet not yet been confirmed, tests suggest (see Chapter 14) that these cysts do not parasitize potato plants. This fact supports their phylogenetic relationships with other *Globodera* spp. as described in Chapter 4, but is also important knowledge for the potato industry, since the possible emergence of new *Globodera* spp. parasitizing potatoes will impact heavily on accessibility to foreign markets and certification of seed potatoes in South Africa.

The description of these species and the development of reliable diagnostic methods (Chapter 6) is of great importance to growers and regulatory services alike, because of the complications in the detection of the quarantine nematode, *G. rostochiensis* that could arise due to the presence of these cysts in regulatory samples from potato-producing areas.

Literature

BEHRENS, E. (1975). *Globodera* Skarbilovic, 1959, eine selbstandige Gattung in der Unterfamilie Heteroderinae Skarbilovic, 1947 (Nematoda: Heteroderidae). *Vortagstagung zu Aktuellen Problemen der Phytonematologie am 29.5.1975 in Rostock. Manuskriptdruck der Vortrage. Biologische Gesellschaft der DDR, Setion Phytopathologie und Universitat, Rostock*, pp. 12-26.

COBB, G.S. & TAYLOR, A.L. (1953). *Heterodera leptonepia* n. sp., a cyst-forming nematode found in soil with stored potatoes. *Proceedings of the Helminthological Society of Washington* 20, 13-15

EROSHENKO, A.S. & KAZACHENKO, I.P. (1972). [*Heterodera artemisiae* sp. Nematode: Heteroderidae) - a new species of cyst-forming nematode from the Primorsk Territory.] *Parazitologija* 6, 166 – 170.

EVANS, K. & ROWE, J.A. (1998). Distribution and economic importance. In: Sharma, S.B. (Ed.). *The Cyst Nematodes*. Dordrecht, The Netherlands, Kluwer Academic Publishers, pp.1-30.

FELSENSTEIN, J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* 39, 783-791.

FERRIS, V.R., KRALL, E., FAGHIHI, J. & FERRIS, J.M. (1999) Phylogenetic relationships of *Globodera millefolii*, *G. artemisiae*, and *Cactodera salina* based on ITS region of ribosomal DNA. *Journal of Nematology* 31, 498-507.

FILIPJEV, I.N. & SCHUURMANS STEKHOVEN, J.H. (1941) *A manual of agricultural helminthology*. Leiden, The Netherlands, E.J. Brill, xv + 878 pp.

FLEMING, C.C. & POWERS, T.O. (1998). Potato cyst nematode diagnostics: morphology, differential hosts and biochemical techniques. In: Marks, R.J. & Brodie, B.B. (Eds.). *Potato Cyst Nematodes. Biology, Distribution and Control.* Wallingford, UK, CAB International, , pp.91 – 114.

FRANCO, N.F., CID DEL PRADO VERA, I. & LAMOTHE-ARGUMEDO, R. (2000). *Globodera bravoae* sp. n. (Tylenchida: Heteroderidae) from Mexico. *International Journal of Nematology* 10, 169-176.

GOLDEN, A.M. & KLINDIĆ, O. (1973). *Heterodera achilleae* n. sp. (Nematoda, Heteroderidae) from yarrow in Yugoslavia. *Journal of Nematology* 5: 196-201.

GRENIER, E., FOURNET, S., PETIT, E. & ANTHOINE, G. (2010). A cyst nematode 'species factory' called the Andes. *Nematology* 12(2), 163-169.

HANDOO, Z.A., CARTA, L.K., SKANTAR, A.M. & CHITWOOD, D.J. (2012). Description of *Globodera ellingtonae* n. sp. (Nematoda: Heteroderidae) from Oregon. *Journal of Nematology* 44, 40–57.

KATOH, K., KUMA, K., TOH, H. & MIYATA, T. (2005). "MAFFT version 5: Improvement in accuracy of multiple sequence alignment". *Nucleic Acids Research* 33, 511-518.

KIRJANOVA, E.S. & BORISENKO, A.V. (1975). [A cyst forming nematode – *Heterodera mali* sp. n. – a new species of parasite of apple trees in the Kazakhstan.] *Parasitoloyiya* 9, 335-338.

KIRJANOVA, E.S. & KRALL, E.L. (1971). [*Plant-parasitic nematodes and their control Volume II.*] Leningrad, USSR, Nauka Publishers Leningrad Section, 522 pp.

KLEYNHANS, K.P.N. (1998). Potato cyst nematodes (*Globodera* species) in Africa. In: Marks, R.J. & Brodie, B.B. (Eds). *Potato cyst nematodes. Biology, Distribution and Control.* Wallingford, UK, CAB International, pp. 347-351.

KNOETZE, R., MALAN, A.P. & MOUTON, C. (2006). Differentiation of South African potato cyst nematodes (PCN) by analysis of the rDNA internal transcribed spacer region. *African Plant Protection* 12, 103 – 110.

KNOETZE, R. SWART, A & TIEDT, L.R. (2013). Description of *Globodera capensis* n. sp. (Nematoda: Heteroderidae) from South Africa. *Nematology* 15, 233-250.

LAX, P. & DOUCET, M.E. (2002). New process for preparing heteroderid cysts for scanning electron microscopy. *Nematology* 4, 445 – 447.

LOWNSBERY, B.F. & LOWNSBERY, J.W. (1954). *Heterodera tabacum*, a parasite of solanaceous plants in Connecticut. *Proceedings of the Helminthological Society of Washington* 21, 43-46.

MADANI, M., SUBBOTIN, S.A., WARD, L.J., LI, X. & DE BOER, S.H. (2010). Molecular characterization of Canadian populations of potato cyst nematodes, *Globodera rostochiensis* and *G. pallida* using ribosomal nuclear RNA and cytochrome b genes. *Canadian Journal of Plant Pathology* 32, 252–263.

MANDURIC, S. & ANDERSSON, S. (2004). The identity of a Swedish *Globodera* (Nematoda: Heteroderidae) population, following comparisons with known populations of

G.artemisiae (Eroshenko & Kazachenko, 1972) Behrens, 1975. *Russian Journal of Nematology* 12, 39-44.

MULVEY, R.H. & STONE, A.R. (1976). Description of *Punctodera matadorensis* n. gen., n. sp. (Nematoda: Heteroderidae) from Saskatchewan, with lists of species and generic diagnosis of *Globodera* (n. rank), *Heterodera* and *Sarisodera*. *Canadian Journal of Zoology* 54, 772-785.

NEI, M. & KUMAR, S. (2000). Molecular Evolution and Phylogenetics. Oxford University Press, New York.

NETSCHER, C. & SEINHORST, J.W. (1969). Propionic acid better than acetic acid for killing nematodes. *Nematologica* 15, 286.

PICARD, D., SEMPERE, T. & PLANTARD, O. (2008). Direction and timing of uplift propagation in the Peruvian Andes deduced from molecular phylogenetics of highland biotaxa. *Earth and Planetary Science Letters* 271, 326–336.

SABO, A, REIS, L.G.L., KRALL, E., MUNDO-OCAMPO, M., & FERRIS, V.R. (2002). Phylogenetic relationships of a distinct species of *Globodera* from Portugal and Two *Punctodera* species. *Journal of Nematology* 34, 263–266.

SEINHORST, J.W. (1959). A rapid method for the transfer of nematodes from fixative to anhydrous glycerin. *Nematologica* 4, 67-69.

SIDDIQI, M. R. (2000). *Tylenchida parasites of plants and insects*, 2nd edition. Wallingford, UK, CABI Publishing, xiii + 833 pp.

SIRCA, S. & UREK, G. (2004). Morphometrical and ribosomal DNA sequence analysis of *Globodera rostochiensis* and *Globodera achilleae* from Slovenia. *Russian Journal of Nematology* 12, 161-168.

SKANTAR, A.M., HANDOO, Z.A., CARTA, L.K. & CHITWOOD, D.J. (2007). Morphological and molecular identification of *Globodera pallida* associated with potato in Idaho. *Journal of Nematology* 39, 133-144.

SKARBILOVICH, T.S. (1947) [Revision of the systematics of the nematodes of the nematode family Anguillulidae Baylis and Daubney, 1926.] *Doklady Akademii Nauk SSSR* 57: 307-309.

SKARBILOVICH, T.S. (1959). On the structure of the systematics of nematodes of the order Tylenchida Thorne, 1949. *Acta Parasitologica Polonica* 7, 117-132.

STONE, A.R. (1973b). *Heterodera pallida*. *CIH Descriptions of plant parasitic nematodes.* Set 2. No. 17. Farnham Royal, UK, Commonwealth Agricultural Bureaux.

STURHAN, D. (1984). Nachweis des Schafgarbenzystennematoden *Globodera millefolii* (Kirjanova & Krall) für die Bundesrepublik Deutschland. *Nachrichtenbl des Deutschen Pflanzenschutzdienstes* 36, 81–82.

STURHAN, D., WOUTS, W.M. & SUBBOTIN, S.A. (2007) An unusual cyst nematode from New Zealand, *Paradolichodera tenuissima* gen. n., sp. n. (Tylenchida: Heteroderidae). *Nematology*, 9, 561-571.

SUBBOTIN, S.A., CID DEL PRADO VERA, I., MUNDO-OCAMPO, M. & BALDWIN, J.G. (2011). Identification, phylogeny and phylogeography of circumfenestrate cyst nematodes

(Nematoda: Heteroderidae) as inferred from analysis of ITS-rDNA. *Nematology*. 13(7), 805-824.

SUBBOTIN, S.A, MUNDO-OCAMPO, M. & BALDWIN, J.G. (2010). *Systematics of cyst nematodes (Nematoda: Heteroderidae).* Nematology monographs and perspectives Volume 8A. Brill, Leiden-Boston, xii + 351 pp.

SUBBOTIN, S.A., VIERSTRAETE, A., DE LEY, P., ROWE, J, WAEYENBERGE, L., MOENS, M. & VANFLETEREN, J.R. (2001). Phylogenetic relationships within the cyst-forming nematodes (Nematoda, Heteroderidae) based on analysis of sequences from the ITS regions of ribosomal DNA. *Molecular Phylogenetics and Evolution* 21, 1–16.

TAMURA, K. & NEI, M. (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution* 10, 512-526.

TAMURA, K., PETERSON, D., PETERSON, N., STECHER, G., NEI, M. & KUMAR, S. (2011). MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution* 28, 2731-2739.

TANHA MAAFI, Z., SUBBOTIN, S.A. & MOENS, M. (2003). Molecular identification of cyst-forming nematodes (Heteroderidae) from Iran and a phylogeny based on ITS-rDNA sequences. *Nematology* 5, 99-111.

UEHARA, T., KUSHIDA, A., ITOU, K., NARABU,T. & MOMOTA, Y. (2005). Discrimination of three cyst-forming nematodes of the genus *Globodera* (Nematode: Heteroderidae)

from Japan based on PCR-RFLP of ribosomal DNA. *Applied Entomology and Zoology (Japan)* 40, 537-543.

VRAIN, T.C., WAKARCHUCK, D.A., LÈVESQUE, A.C. & HAMILTON, R.I. (1992). Intraspecific rDNA restriction fragment length polymorphism in the *Xiphinema americanum* group. *Fundamental and Applied Nematology* 15, 563-573.

WOLLENWEBER, H.W. (1923). Krankheiten und Beschädigungen der Kartoffel. *Arbeiten des Forschungsinstitutes für Kartoffelbau*, Heft 7, Paul Parey, Berlin, 56 pp.

WOUTS, W.M. (1984). *Globodera zelandica* n. sp. (Nematoda: Heteroderidae) from New Zealand, with a key to the species of the genus *Globodera*. *New Zealand Journal of Zoology* 11, 129-135.

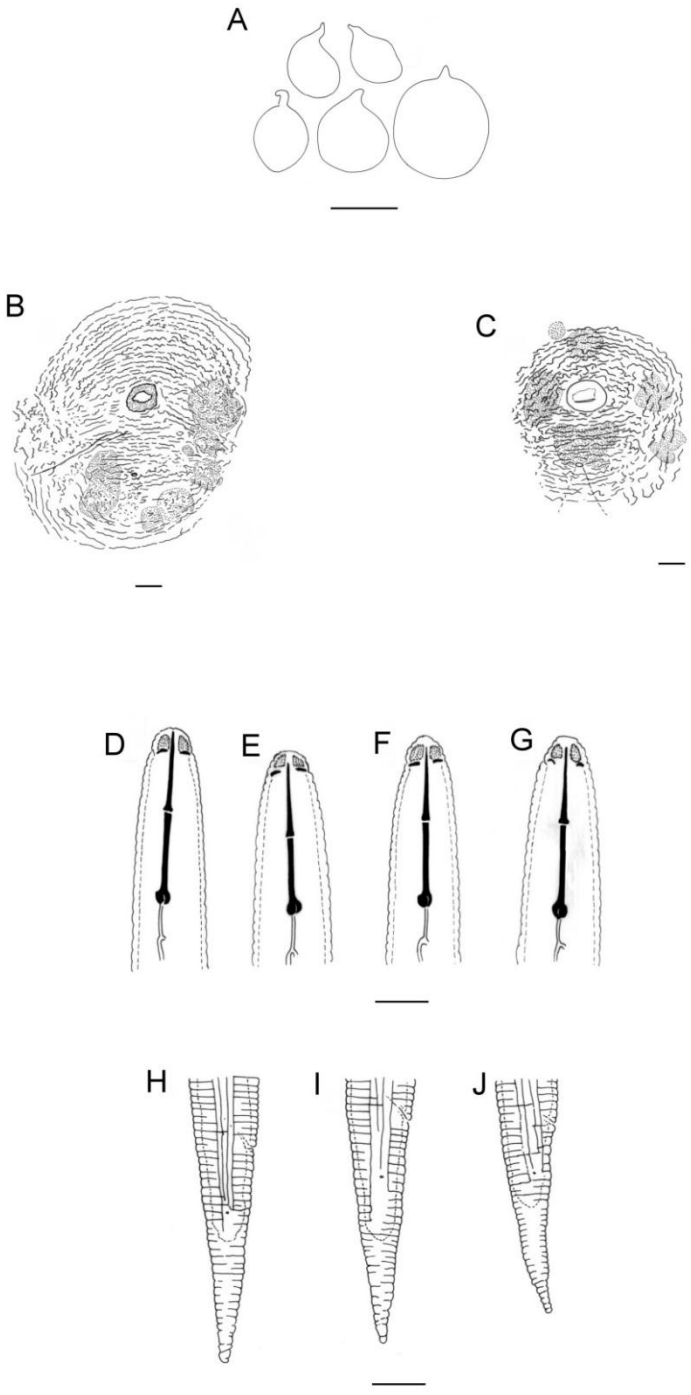

Fig 8.1. Cysts and second stage juveniles of *Globodera capensis* n. sp. A: Whole cysts. B,C: Terminal areas of showing subcuticular bullae and cuticular ridges between vulval basin and anus; D-G: Heads of different J2; H-J: Tails of different J2. (Scale bars: A = 500 µm; B-J = 10 µm) (Knoetze *et al*, 2013).

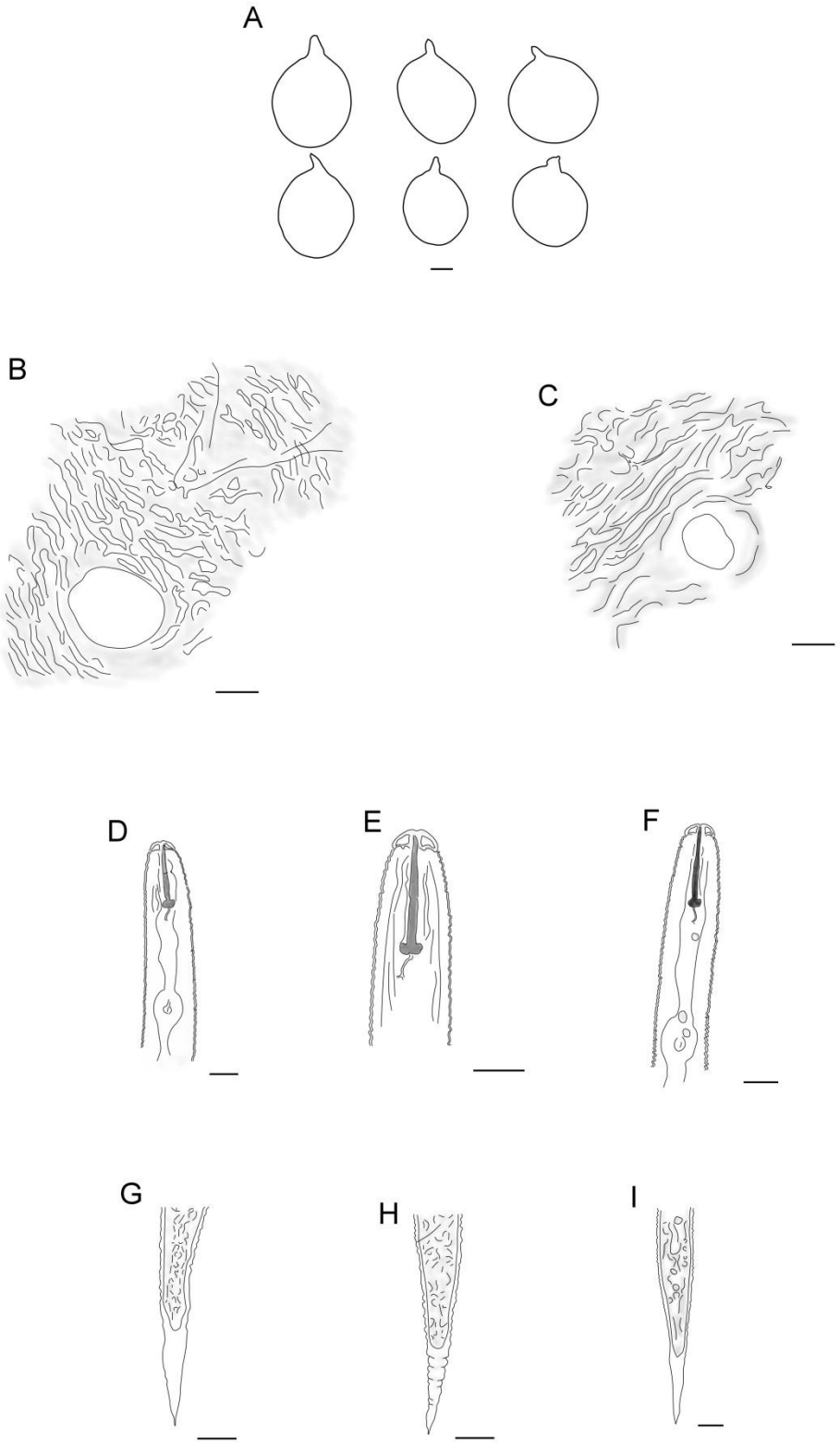

Fig 8.2. Cysts and second stage juveniles of *Globodera* sp. SK18. A: Whole cysts; B, C: Terminal areas of different cysts showing cuticular ridges between vulval basin and anus; D-F: Heads of different J2; G-I: Tails of different J2.(Scale bars: A = 100 µm; B-I = 10 µm)

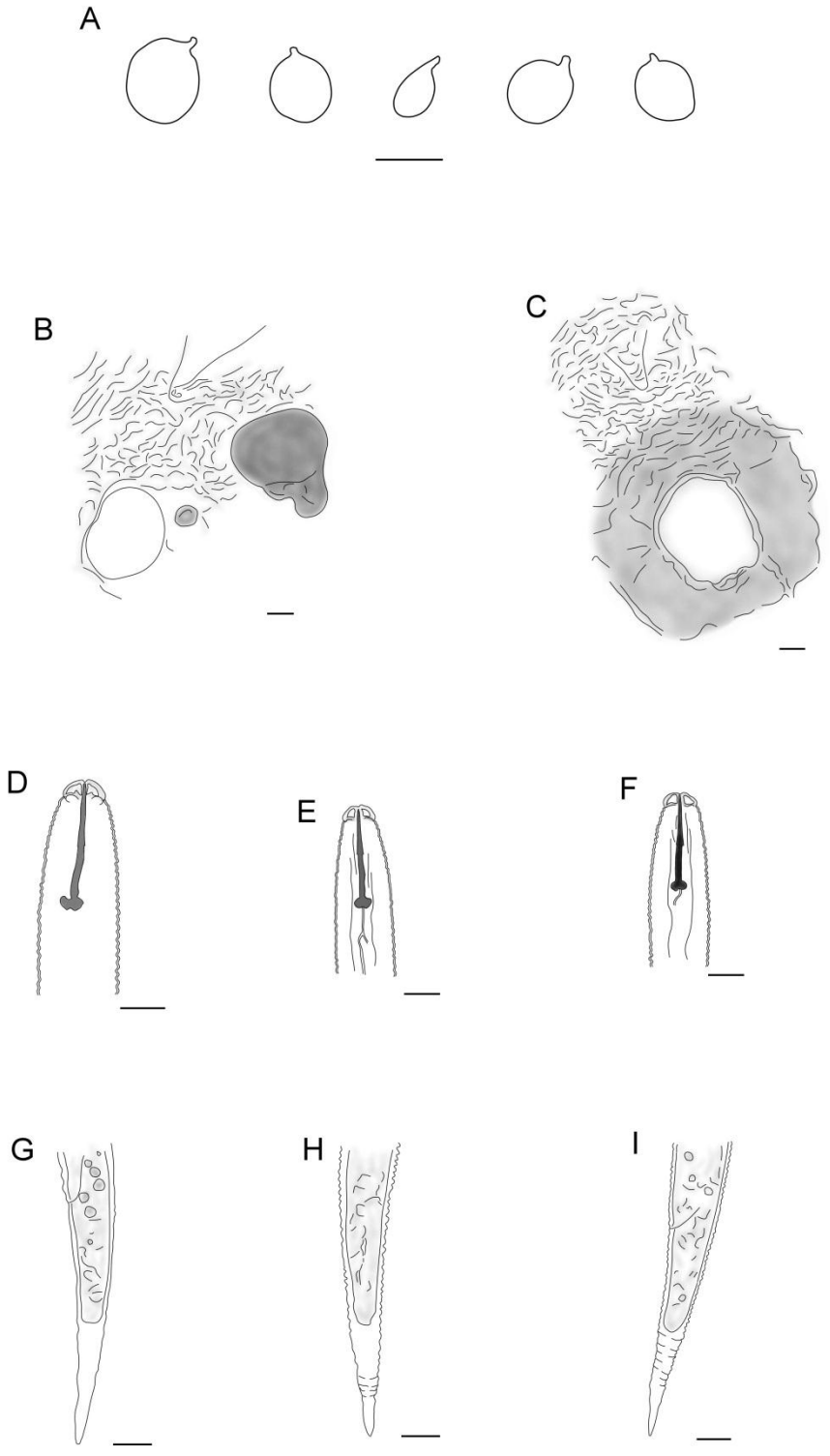

Fig 8.3. Cysts and second stage juveniles of *Globodera* sp. WK1. A: Whole cysts; B, C: Terminal areas of different cysts showing subcuticular bullae and cuticular ridges between vulval basin and anus; D-F: Heads of different J2; G-I: Tails of different J2. (Scale bars: A = 500 µm; B-I = 10 µm)

Chapter 9

The effect of storage temperature and field conditions on the viability and hatching of South African populations of *Globodera rostochiensis* (Nematoda: Heteroderidae)

Introduction

The success of *Globodera rostochiensis* (Wollenweber, 1923) Skarbilovich, 1959 as an agricultural pest is partly due to their ability to survive adverse conditions for extended periods of time (Turner & Evans, 1998). *G. rostochiensis* has been detected in some of the main potato-producing areas in South Africa, including the Sandveld, Ceres, South-western Cape, Eastern Cape and Gauteng areas (Chapter 3). Severe climatic conditions occur in these regions, ranging from summer temperatures above 40°C in the Sandveld to winter temperatures below freezing in the Ceres area. To determine the extent to which the populations of *G. rostochiensis* have adapted to local conditions, we need to evaluate the ability of the cysts of local populations to survive in the soil, enabling us to make informed decisions for the management of these nematodes.

The life cycle of potato cyst nematodes (PCN) comprises the egg, juveniles and adults. The embryos develop within the egg as far as the second juvenile stage (J2) while still within the body of the female. The female then dies and transforms into a protective cyst containing 200-500 eggs. After fertilisation and subsequent cyst formation, the unhatched, encysted J2 remain dormant. Dormancy has been separated into quiescence and diapause (Perry, 1989).

Once the J2 have developed inside the cysts, they enter diapause, during which time they cannot be stimulated to hatch. Diapause generally terminates before the next crop and most juveniles only enter diapause during their first year (Turner & Evans, 1998). Quiescence may follow after diapause has ended.

In the absence of a host plant, some juveniles will hatch spontaneously when soil temperature and moisture content are suitable. In temperate climates, the PCN populations decline by about 30% per year in the absence of a host crop (Evans & Stone, 1977). In cold soils the annual decline due to hatch may be as low as 18% (Grainger, 1964), whereas in soils above 30°C it can be as much as 95% (Schluter, 1976). Yearly decline also depends on the humidity of the soil. In dry soil the decline may be less than 20%, in moist soils as much as 50% (Oostenbrink, 1950). Soil type can also affect hatching with up to 60% hatching occurring in sandy soils (Cole & Howard, 1962b). They also found that 4 years of resistant potatoes have reduced the egg population to about 1% of the initial value, whereas 4 years of non-host crops have only reduced it to about 25%. Under colder natural conditions in Norway, storage in soil gave an annual decline of 4% (Øydvin, 1978), while in southern Italy, few nematodes survive the high summer soil temperatures (Evans & Stone, 1977). Under European conditions, soil infestations of PCN may persist for 20 to 30 years in the absence of a suitable host (Turner, 1996).

Soil moisture plays an important part in determining the annual decline of PCN infestations, this being greater on drier soils (Grainger, 1964). Natural decline of *G. rostochiensis* depends to some degree on the kind of crop grown between potato plantings (Grainger, 1964), and regular cultivation of infested land may accelerate rate of decline (Turner, 1996). Cysts are also attacked by other organisms, like fungi (Kerry & Crump, 1977), bacteria (Stirling, 1984), ricketsias, mites and other nematodes (Mankau, 1980).

In Bolivia, 0.25 eggs gram^{-1} of soil was found 10 years after the last potato crop, but no cysts were found after 15 years, while in Peru no cysts were found after 5 years of fallow (Turner & Evans, 1998). Marshall (1998) found that *G. rostochiensis* declines annually between 45% and 61% in New Zealand in the absence of a host, depending on the soil type. Turner (1996) monitored 48 fields for PCN from last potato production, annually for up to 8 years, as well as fields that have been out of production for 6 years, assessing decline rates between 7 and 13 years. Results were expressed as cysts per sample, eggs per cyst and eggs per g soil. Regression analysis of data indicated that overall PCN decline in agricultural soils in Northern Ireland appeared to be erratic due to variable cyst ages and high standard errors when sampling low population levels. LaMondia (1987) counted viable eggs per cyst by crushing cysts to measure the effects of potato root diffusate on populations and measured viable eggs per cubic centimeter of soil to estimate naturally infested soil. Whitehead (1995) counted viable eggs per gram soil by crushing cysts and releasing the eggs with a stirrer and counting the eggs in the suspension. He concluded that counting 'full' cysts probably underestimate decline. Devine *et al*. (1999) investigated the rate and mechanisms of PCN population decline by directly measuring changes in both the percentage of non-viable eggs present in the population and in the rate of spontaneous hatch. The decline of viable eggs per gram soil was found to be 57% in one site (first year in rotation) and 40 % for another site (second year in rotation). These decline rates are considerably higher than 30% commonly used for modeling purposes. The decline was found to be due to a combination of spontaneous hatch and in-egg mortality. The rates of in-egg mortality increased with soil temperature. Temperature was also found to influence spontaneous hatch. The changes in the viable PCN population size involved both in-egg mortality, starting at 4°C and spontaneous hatch at temperatures above 10°C. The study also assessed the possible role of culturable hydrolase-producing bacteria in the degradation of non-viable eggs. At Bon Accord, South

Africa the population density of *G. rostochiensis* in one field decreased from 16.3 eggs g^{-1} soil to 2.1 eggs g^{-1} soil over 2 years under non-host crops. In another field, planted to non-host crops for 5 years, the residual population decreased from 30.1 eggs g^{-1} soil to 0.9 eggs g^{-1} soil over 3 years of non-host crops (Kleynhans, 1988). Le Roux (2000) conducted field trials in the Ceres area, South Africa, to study the life cycle, epidemiology and control of *Globodera rostochiensis* under hot South African conditions. In a fallow treatment she observed a 60% decrease in viability in 6 summer months. She measured viable eggs per cyst and viable eggs per 100 cc soil. The maximum ambient temperature at the Ceres trial was recorded as 17-34°C and the minimum ambient temperature was 4-17°C. The maximum soil temperature was 21 - 39°C. Munir *et al.* (2009) found that eggs in cysts can survive for some time at high temperatures, provided they are dry, but lose hatching ability at these temperatures when moist. To test the survival of cysts at high field temperatures they buried a nylon bag of cysts in the soil for 8 weeks together with a temperature data logger.

In the presence of root diffusates from a host crop, 60-80% of juveniles can be stimulated to hatch, with more hatching in sandy soils than in peat (Jones, 1970). As part of their survival strategy, hatching never reaches 100%, no matter which stimulating conditions are present. Unhatched J2's are quiescent until stimulated to hatch by root diffusates emanating from the host-plant root, in this case plants within the family Solanaceae. Root diffusates from both susceptible and resistant varieties of potato stimulate hatching of PCN (Clarke & Perry, 1977). Hatching of PCN in root diffusates collected from twenty-five potato cultivars indicated that the diffusates from most cultivars hatched *G. rostochiensis* well, some hatched *G. pallida* well and some hatched it poorly (Evans, 1983). Initiation of hatching is rapid, as exposure to potato root diffusates (PRD) for as short as 5 min triggers substantial hatching of *G. rostochiensis* (Perry & Beane, 1982). The speed of juvenile emergence in PRD differs

between populations of the same species (Whitehead, 1992). The rates of hatch and emergence of J2s from cysts of *G. rostochiensis* were found to be dependent on hatching factor concentration *in vitro* and in the field (Devine & Jones, 2000). The response of the PCN populations *in vitro* to hatching agents was found to be dependent on the physiological state of the egg/juvenile complex, with egg populations in diapause responding to the presence of natural and artificial hatching factors by exhibiting increased in-egg mortality but not increased hatch (Devine & Jones, 2001). Ryan & Jones (2003) found that leachate from sterile-grown plants exhibit significantly less PCN hatching activity than the leachate from non-sterile grown plants. Their results suggested that soil micro-organisms play an important role in the production of hatching chemicals. The effects of cyst components of *G. rostochiensis* on *in vitro* hatching of juveniles were evaluated by Pridannikov *et al.* (2007). Homogenates of cyst walls or rinsates of either cyst walls or eggs each significantly increased hatch of juveniles when compared with sterile distilled water controls. Turner *et al*. (2009) found that, whilst a *G. rostochiensis* Ro1 population showed no variation in hatch, other populations displayed differences in hatch in the presence of PRD from different wild potato clones.

In Western Europe, the optimum temperature for hatching in *G. rostochiensis* is close to 20°C with a lower limit of about 10°C and a higher limit of about 27°C (Mulder, 1988b). Ellenby & Smith (1975) found that there was a profound difference between populations from Newcastle and Ayrshire, the latter showing an adaptation to local cultural practices in its hatching behavior at low temperatures. Because potatoes in this area are planted and lifted early, emergence from cysts is large at 10°C for the Ayrshire population, but not for the Newcastle one. Hatching and development of five populations of PCN were studied at different temperatures by Franco (1979). He found that *G. pallida* populations hatched and

reproduced more at lower temperatures than *G. rostochiensis* populations, but *G. rostochiensis* was more successful at temperatures above 20°C. Robinson *et al.* (1987) found that maximum hatch occurred between 15 to 20°C for both species with a significant peak at 20°C for *G. rostochiensis*. *G. rostochiensis* was significantly less active at 25°C than at other temperatures. The optimum hatching temperature for a Western-Australian population of *G. rostochiensis* was 22°C in picrolonic acid (Stanton & Sartori, 1990).

Hominick *et al*. (1985) adopted a strict protocol to establish the presence of a diapause, eliminating operator bias, population variability and environmental influences. They found that under conditions optimal for hatching, juveniles emerged more slowly and hatching was more prolonged in the autumn and early winter after harvest than in the following spring and summer. They identified three effects that must be considered when assessing the hatch of *G. rostochiensis* cysts: 1) the degree of maturation. Emergence from females harvested while still yellow was greater than from cysts harvested after turning brown. Ellenby & Smith (1967) concluded that cyst maturation is accompanied by an endogenous process of hatch inhibition that can result in dormancy; 2) A first season diapause which is apparently obligate and is observed when mainly new cysts are used 3) A seasonal dormancy influenced by conditions in the soil but absent if cysts are stored under constant conditions. Hominick (1986) assessed hatching of *G. rostochiensis* grown in complete darkness or in different light regimes. Rearing the cysts in darkness prolonged diapause, while emergence from cysts grown on constant light was much more rapid than from cysts grown in other conditions. Salazar & Ritter (1993) found that pre-hatch storage of cysts modifies the hatching rhythm in PCN, and the effect of pre-hatch storage conditions is influenced by the photoperiod at the time of development of females. Juveniles from cysts produced under increasing day length conditions hatched with no apparent limitation, while the shorter days were more variable and

lower hatching rates were recorded. Cyst progenies obtained under a short day regime had low hatching rates when stored at 13°C and no hatching at 22°C, while those obtained from moderate short days increased with the storage temperature from 13°C to 22°C. Long day conditions produced high hatching rates. It seems that increasing day length during the life cycle prevented the establishment of diapause. Muhammad & Evans (1997) investigated hatching as an indicator of diapause by hatching both old and new cysts for a 12 month period in PRD. New cysts were collected from cultures after 3 months and either used as new cysts or stored outdoors for one calendar year. After the completion of hatching, cysts were broken open and the number of viable eggs counted. The cumulative percentage of emergence in new cysts increased from less than 50% in January and April to more than 80% in October. Their results clearly indicate a different emergence pattern between old and new cysts, lending support to the hypothesis that new cysts experienced a form of dormancy which had been lost from the old cysts during their year of storage outdoors. McKenna & Winslow (1972) found that different pathotypes all hatched more rapidly as the season advanced, although it was always incubated at the same temperature (25°C).

Janssen *et al*. (1987) performed artificial hatching by cutting cysts in halves with a scalpel and incubating in root diffusates. This favoured the hatchability to such an extent that it enabled the propagation of three to five generations a year in pots. The water regime (avoiding desiccation) in the cysts and the cutting of the cyst wall was essential in bypassing the natural diapause. When cysts were left to desiccate the number of artificially hatched larvae was strongly reduced. Been & Schomaker (2001) identified and analysed sources of variability in hatching tests and presented some solutions. A method was developed to conduct hatching tests using inert materials to minimise the total variation at the end of the test. Hatching tests were carried out to increase reliability, optimise the method and limit the

amount of work. Desgarennes *et al.* (2006) was able to classify cysts as young, mature and old on the basis of cyst wall colour, helping them to understand population status. Also important are differences in the stages that occur within cysts, as they are linked to color differences. The greatest degree of hatching was observed in mature cysts.

In this study we will study the effect of local conditions and storage at different temperatures on the viability and hatching of South African populations of *G. rostochiensis* over time will be investigated.

Material and methods

POPULATION DECLINE IN FIELD PLOTS INFECTED WITH *G. ROSTOCHIENSIS*

Infected plots in five geographically separate locations were selected for monitoring. They originate from unique locations in the Sandveld, Ceres, South-Western Cape, Eastern Cape and Gauteng potato-producing areas. The plots are listed in Table 9.1.

Samples were collected by taking 50 core samples, distributed over a maximum area of 4 ha. Areas of more than 4 ha was subdivided into units of 4 ha or less. To ensure systematic distribution of core-samples, sampling was done in a zigzag pattern across the field. Core-samples were obtained by using a soil sampling auger of the "cheese-sampler" type with half-cylindrical blade 20-30 cm long and 20-25 mm wide. Sampling was done to a depth of 10 – 20 cm, resulting in a final sample of approximately 1750 cc. Although efforts were made to sample the plots annually, constraints were experienced with regard to availability of manpower and resources, which caused sampling to be rather irregular (Table 9.1). Sampling was ceased on a particular plot if no more viable cysts were detected.

Cysts were extracted with a Seinhorst cyst elutriator (Seinhorst, 1964). Cysts were washed through a 840 µm aperture sieve over a 250 µm aperture sieve, collecting into the latter. The content of this sieve was transferred to a piece of filter paper in a funnel and left to dry. The dried debris and cysts were examined under a stereomicroscope. Three subsamples from each plot were analysed at each sampling time.

CULTURING OF CYSTS

Cysts used in experiments were obtained from glasshouse cultures. Potato tubers (cv. BP1) were planted in washed sand in plastic pots with the drainage holes covered with milk filters. Soaked, viable cysts were crushed and eggs and juveniles were added to the pots. The plants were maintained at a cycle of 14h light and 10h dark at 25°C in a glasshouse. The plants were removed from the glasshouse after 12 weeks and new cysts were extracted using a Seinhorst cyst elutriator (Seinhorst, 1964).

EFFECT OF STORAGE TEMPERATURE ON IN-EGG MORTALITY AND *IN VITRO* HATCH OF DIFFERENT POPULATIONS OF *G. ROSTOCHIENSIS*

Cysts from three geographically separate locations (S, P and H in Table 9.1) were extracted from cultures, surface dried and stored at 4 °C before use. Cysts were used for incubation within 3 days from storage. Batches of 15 cysts were placed in plastic vials, which were incubated in the dark at 20°C, 30°C and 40°C. A vial from each temperature was removed at 3, 6, 9, 12, 15, 18, 21 and 24 months and used for hatching tests and the determination of

viability. One batch of 15 cysts was also evaluated directly after extraction from cultures, for use as benchmark values in the hatching and viability tests.

EFFECT OF FIELD CONDITIONS ON IN-EGG MORTALITY AND *IN VITRO* HATCH OF DIFFERENT POPULATIONS OF *G. ROSTOCHIENSIS*

Cysts from five geographically separate locations (Table 9.1) were extracted from cultures, surface dried and stored at 4 °C before use. Cysts were used for incubation within 3 days from storage. Batches of 20 cysts were placed into small bags, manufactured from 90 µm aperture nylon mesh. The bags were buried at 20 cm depth in field soil on location at the Plant Quarantine Station in Stellenbosch (S 33° 56.885'; EO 18° 49.594'), as well as at a location in the Sandveld (S 32° 31.132'; EO 18° 30.873'), an important potato producing area in South Africa. One bag from each population was removed at 6, 12, 18 and 24 months and used for hatching tests and the determination of viability of eggs and the amount of spontaneous hatch. One batch of 15 cysts was also evaluated directly after extraction from the cultures, for use as benchmark values in the hatching and viability tests.

SOIL TEMPERATURES

Data loggers (HOBO® Pendant from Onset® Computer Corporation) were buried at the same locations as the bags with cysts to record soil temperatures during the incubation periods. The data was analysed with HOBOware® Pro Graphing & Analysis Software (Onset® Computer Corporation).

POTATO ROOT EXUDATES

Potato root exudates (PRD) were obtained by removing one month old potato plants (cv. BP1) from the pot and rinsing the roots to remove soil particles. The plants were then kept at 25°C with the roots in 50 ml distilled water in glass beakers for 24 h. The diffusates were then passed through a 90 µm sieve and stored at 4°C. Before use, the diffusate was sterilised by passing through a 0.45 µm pore cellulose-acetate filter.

HATCHING TESTS

Two-hundred microliters of purified water (Millipore) was dispensed into the wells of a 96-well ELISA plate. The cysts from each treatment were placed into the wells (one per well) and incubated at 20°C. After seven days the hatched juveniles were counted using a stereomicroscope and the cysts were transferred to new wells, this time containing 200 µl potato root exudate. Hatched juveniles were counted at 7 day intervals for a maximum of ten weeks. Cysts were transferred to fresh PRD at the end of each 7 day period. After ten weeks, the cysts were used to determine the viability of unhatched eggs. Percentage hatch was calculated as the ratio of juveniles to the original number of eggs in each cyst. Cumulative hatching percentages over the ten week period were also calculated for the field conditions experiment.

IN-EGG MORTALITY AND POPULATION DECLINE

Soaked cysts were placed in 0.05% aqueous solution of Meldola's blue (Shepherd, 1962) for 1 week. For the evaluation of cysts extracted from infested fields, the cysts were crushed in a microcentrifuge tube with a homogeniser and the eggs and juvenile contents were released by vortexing. The eggs were separated from the cyst fragments by washing them through a 90 µm sieve, collecting on a 25 µm sieve. Eggs and juveniles were left in a water suspension for up to 12h and observed in a counting chamber. The decline of the population was estimated by removing two aliquots and counting non-stained (viable) eggs at each sampling time. For the storage temperatures and field conditions experiments, cysts were cut open individually and the eggs were removed. The eggs were then pipetted onto a counting chamber and observed under a research microscope. The viability of the eggs was estimated by counting stained (non-viable) and non-stained (viable) eggs. Spontaneous hatch was estimated by counting empty eggs and deducting the amount of J2's that hatched during the hatching assay.

DATA ANALYSIS

Data were analysed either with a factorial analysis of variance (ANOVA) if more than one factor was involved or with one-way ANOVA if only one factor was involved. In all tests a significance level of 5% was used. If significant interaction was observed in the factorial ANOVA, the interaction effects/means were analysed with Bonferroni multiple comparisons to detect which interaction effects differ. All statistical analyses were done with STATISTICA version 11 (StatSoft Inc. 2012).

Results

POPULATION DECLINE IN FIELD PLOTS INFECTED WITH *G. ROSTOCHIENSIS*

Table 10.1 shows the amount of cysts 100 cc^{-1} and viable eggs 100 cc^{-1} that were recorded from the different plots at different sampling times. Data analysed with one-way ANOVA showed no significant differences between the amount of viable eggs recorded at different intervals for plots S ($F_{(3, 8)} = 0.796$; $P = 0.530$) and H ($F_{(1, 4)} = 5.147$; $P = 0.086$). Data from plot C showed significant differences between the amount of viable eggs 100 cc^{-1} recorded at different intervals ($F_{(3, 8)} = 4.122$; $P = 0.0485$). The decline in the amount of viable eggs for plot S and H were therefore not significant between sampling dates ($P > 0.05$), while the decline of the amount of viable eggs 100 cc^{-1} for plot C were significant between 0 and 24, as well as between 0 and 39 months ($P < 0.05$), but not between 0 and 8 months. The overall decline rate of viable eggs soil was 87.06% for plot S and 83.41% for plot C. Only one sample was taken at plot P, since no viable cysts were detected in the first sample. No cysts could be detected in the second sample of plot G, which was taken 17 months after the first. Only two samples were taken from plot H, but in that period the population exhibited a decline rate of 79.55%.

EFFECT OF STORAGE TEMPERATURE ON *IN VITRO* HATCH OF DIFFERENT POPULATIONS OF *G. ROSTOCHIENSIS*

Data analysed with a factorial ANOVA showed significant differences ($F_{(16, 1115)} = 15.566$; $P = 0.0000$) when comparing the percentage hatch at different temperatures. Figure 9.1 shows

the percentage hatch of juveniles for populations S, P and H, stored at 20°C for 3–24 months. Population S displayed a significant increase in hatch percentage at 9 months (62% ± 30%), when compared to the control at 0 months storage ($P < 0.05$). At 3, 6, 12, 15, 18, 21 and 24 months storage at 20°C, the hatch percentage of S did not differ significantly from the control. Hatch percentages for P were generally less than that of the control, but only significantly so at 3, 6, 9, 15 and 24 months ($P < 0.05$). Hatch percentages for H increased significantly at 6, 15 and 21 months ($P < 0.05$). The hatch percentages for S and H did not differ significantly for their controls at 0 months ($P > 0.05$), enabling us to compare their hatch percentages at the different storage periods. The hatch percentage at 9 months storage was significantly higher ($P < 0.05$) for S. At 6, 12, 15 and 21 months the hatch percentages for H was significantly higher ($P < 0.05$) than that of S. Figure 10.2 shows the percentage hatch of juveniles for populations S, P and H, stored at 30°C for 3 – 24 months. Low hatching percentages with no significant differences ($P > 0.05$) were observed for S. Significant decrease in hatching percentages between the control at 0 months storage and all the other storage periods were observed for P ($P < 0.05$). The hatch percentage at 15 months storage (28% ± 29%) was significantly higher ($P < 0.05$) than that of the control for population H, but not for all the other storage periods. The hatch percentage at 15 months storage for population H was also significantly higher ($P < 0.05$) than the corresponding treatment for the other populations. Figure 10.3 shows the percentage hatch of juveniles for populations S, P and H, stored at 40°C for 3 – 24 months. At 40°C a significant decline in hatching percentages ($P < 0.05$) occurred after the first 3 months for all populations, thereafter very low hatching percentages with no significant differences between them was observed until hatching ceased altogether at 9, 12 and 21 months for P, S and H, respectively. On average, lower hatching percentages (16% ± 21% vs. 6% ± 12%) were observed for all populations at all stages, when

stored at 30°C as opposed to 20°C, but only significant ($P < 0.05$) at 9, 15 and 21 months (Fig. 9.4).

EFFECT OF STORAGE TEMPERATURE ON IN-EGG MORTALITY OF DIFFERENT POPULATIONS OF *G. ROSTOCHIENSIS*

Data analysed with a factorial ANOVA showed significant differences ($F_{(16, 1115)} = 30.173$; $P = 0.0000$) when comparing the percentage viable eggs per cyst at different temperatures. Figure 9.5 shows the percentage viable eggs per cyst for populations S, P and H, stored at 20°C for 3 – 24 months. Population S showed a significant decrease ($P < 0.05$) in the percentage of viable eggs at months 6, 12, 15, 18, 21 and 24. P also showed significant decrease ($P < 0.05$) in the percentage of viable eggs at months 3, 6, 9, 15 and 24. No significant in the percentage of viable eggs were observed for H. An increase of the percentage viable eggs between the control and the rest of the observations for H is attributed to an unexplained high rate of mortality of the specific batch of cysts, since an increase in the amount of viable eggs is highly unlikely. Figure 9.6 shows the percentage viable eggs per cyst for populations S, P and H, stored at 30°C for 3 – 24 months. A significant decrease ($P < 0.05$) in the percentage of viable cysts when compared to the control at 0 months, were observed at all the months storage for both S and P. For H a significant decrease can be observed at months 6, 15, 18, 21 and 24 months, when compared with month 3 (Month 0 not used for reasons explained above). Figure 9.7 shows the percentage viable eggs per cyst for populations S, P and H, stored at 40°C for 3 – 24 months. A significant decrease ($P < 0.05$) in the percentage of viable cysts when compared to the control at 0 months, were observed at all the months storage for S and P, and for H when compared with month 3. For S and P, the

percentage of viable eggs descended to below 10% at 3 months, while for H, the percentage of viable eggs was still high at 3 months (60% ± 31%). The average percentage of viable eggs for all populations was significantly lower (between 19% and 46% lower) from 9 – 24 months storage at 30°C , when comparing to storage at 20°C (Fig. 9.8).

EFFECT OF FIELD CONDITIONS ON *IN VITRO* HATCH OF DIFFERENT POPULATIONS OF *G. ROSTOCHIENSIS*

Data analysed with a factorial ANOVA showed significant differences ($F_{(20, 511)} = 15.556$; $P = 0.0000$) when comparing the percentage hatch from different periods of burial. Figure 9.9 shows the percentage hatch from cysts of different populations at 0 – 24 months. Low hatch percentages (< 20%) were observed for the control at 0 months for all populations, but no significant differences ($P > 0.05$) were observed. At month 6, SS and G both had significantly higher hatch percentages ($P < 0.05$) than the other populations, but did not differ significantly from each other. At month 12, G had significantly higher hatch percentages ($P < 0.05$) than all the other populations, except SS. G and SS had significantly higher hatch percentages ($P < 0.05$) than all the other populations at month 18, but did not differ significantly from each other. . At month 24, low hatch percentages (< 20%) were observed, but there were no significant differences between the populations. Significantly higher hatching percentages ($P < 0.05$) than the control at 0 months were observed at month 6 and 18 for SS and at month 6 and 12 for G, but hatching percentages for the other populations did not differ significantly from their controls at any of the observations. These populations also displayed low hatch percentages throughout the period of observation.

EFFECT OF FIELD CONDITIONS ON IN-EGG MORTALITY AND SPONTANEOUS HATCH OF DIFFERENT POPULATIONS OF *G. ROSTOCHIENSIS*

Data analysed with a factorial ANOVA showed significant differences ($F_{20, 511)} = 4.0879$; $P = 0.0000$) when comparing the percentage viable eggs per cyst. When the percentage of viable eggs per cyst is considered for the populations buried in field soil, a decrease can be observed for all the populations over the 24 month period (Fig. 9.10). Significant decreases were observed at 24 months for S, 6 and 24 months for SS, 12 and 18 months for P and 24 months for G. Low levels of viable eggs were recorded for C, P and H throughout the observation period.

Data analysed with a factorial ANOVA showed significant differences ($F_{20, 511)} = 6.2922$; $P = 0.0000$) when comparing the spontaneous hatch of eggs per cyst. The percentage of spontaneous hatch increased significantly ($P < 0.05$) after 24 months for S, after 18 and 24 months for P and after 12, 18 and 24 months for G (Fig 9.11). The insignificant increase overall in hatched eggs from C and H can be attributed the high mortality rate of eggs in these populations from the onset. The highest in increase in spontaneous hatch was observed in G, rising from less than 1% to almost 40% in 24 months.

Although the percentage of dead eggs per cyst (Figure 9.12) increased for all the populations over time, none of the increases were significant ($P > 0.05$).

SOIL TEMPERATURES IN THE DIFFERENT LOCATIONS

Soil temperature fluctuations for 12 months (April 2012 to March 2013) at the Stellenbosch location are shown in Figure 9.13. The average temperature during this period was 19.9 ± 6.7

°C, with a minimum of 9.5°C and a maximum of 35.8°C. Soil temperature fluctuations for 12 months (October 2011 to September 2012) at the Sandveld location are shown in Figure 10.14. In this case the average temperature was 21.0 ± 7.2 °C, with a minimum of 6.7°C and a maximum of 36.4°C.

Discussion

Results from the hatching tests for cysts from different populations stored at 20°C suggest that diapause did occur in new cysts. Comparison of populations S and H suggest that the diapause lasted longer (9 months) for S than for H (6 months). However hatch decreased again after the 9 months peak for S, while for H it increased again at 15 and 21 months, suggesting that storage at 20°C had more of a detrimental effect on S than H. A decrease in the viability of eggs in the S and P populations, but not H, after storage at 20°C suggest that H survived better at this temperature. When stored at 30°C, hatching decreased significantly for P after just 3 months storage, while for C the decrease was not significant. This was not due to these populations not overcoming diapause but rather because of the decrease in viable eggs in the cysts.

The percentage of viable eggs, although decreasing, stayed above 40% up to 18 months of storage at 30°C for H, explaining why hatching increased in this population, because hatching could continue normally after diapause was overcome. The fact that the viability of eggs for S and P declined to below 10% in a shorter time than for H, suggests H can survive longer at this temperature. Sharp decline in viable eggs also caused low hatch after just 3 months storage at 40°C. These results suggest that storage of cysts at higher temperatures does not

directly influence the hatching ability of eggs, but that the hatch is decreased because of the increased mortality of eggs in the cyst.

Results from the burial of cysts from SS and G in field soil also showed that diapause was overcome within 6 months under field conditions. After the diapause was overcome, there was no significant decrease in the hatching percentage of cysts buried in the soil for 2 years. The lower hatching observed in some of the populations can be attributed to their low percentage of viable eggs in the cysts from the onset and the fact that their viability declined to very low levels within 6 – 12 months. The burial of cysts from the different populations for 2 years caused a decline in viable eggs in these cysts, but suggests that the cysts will be able to survive for much longer in these soils. This observation was confirmed by observation of infested plots where viable cysts remain in the soil after more than 3 years of the plots being fallow. Since no significant increase in the amount of dead eggs was observed, but an increase in spontaneous hatch was observed in most of the populations, spontaneous hatch is deemed to be the main contributor to the decline of viability of these cysts in the time period of the study.

Recording of soil temperatures in two locations have shown that the average temperature at 20 cm depth is not as high as was believed at the beginning of this study. These measurements, together with the results from hatching and viability tests suggest that *G. rostochiensis* might be able to survive without a host for longer periods in the extreme climates of the Sandveld and Ceres areas, than was previously believed. Suggestions that the quarantine period for infested plots in these areas can be shortened were therefore premature and unsubstantiated.

Literature

BEEN, T. H. & SCHOMAKER, C. H. (2001) Ways to improve the accuracy of hatching tests for *Globodera* spp. with special emphasis on nematicide trials. *Nematology* 3, 773-795.

CLARKE, A. J. & PERRY R.N. (1977) Hatching of cyst nematodes. *Nematologica* 23, 350-368.

COLE, C.S. & HOWARD, H. W. (1962b) The effect of growing resistant potatoes on a pota-root eelworm population – a microplot experiment. *Annals of Applied Biology* 50, 121-127.

DESGARENNES, D., CARRIÓN, G., NÚÑES-SANCHEZ, A. E. & NÚÑES-CAMARGO, M. C. (2006). Distribution of stages and *in vitro* larval hatching in *Globodera rostochiensis* cysts. *Nematropica* 36, 251-260.

DEVINE, K.J., DUNNE, C., O'GARA, F. & JONES, P. W. (1999). The influence of in-egg mortality and spontaneous hatching on the decline of *Globodera rostochiensis* during crop rotation in the absence of the host potato crop in the field. *Nematology* 1, 637-645.

DEVINE, K. J. & JONES, P. W. (2000) Response of *Globodera rostochiensis* to exogenously applied hatching factors. *Ann. appl. Biol.* 137, 21-29

DEVINE, K. J. & JONES, P. W. (2001) Effects of hatching factors on potato cyst nematode hatch and in-egg mortality in soil and *in vitro.Nematology* 3, 65-74.

ELLENBY, C. & SMITH, L. (1967). Emergence of larvae from new cysts of the potato root eelworm *Heterodera rostochiensis*. *Nematologica* 13, 273-278.

ELLENBY, C. & SMITH, L. (1975). Temperature adaptation in the potato cyst nematode *Heterodera rostochiensis*. *Nematologica* 21, 114-115.

EVANS, K. (1983) Hatching of potato cyst nematodes in root diffusates collected from twenty-five potato cultivars. *Crop Protection* 2, 97-103.

EVANS, K. & STONE, A. R. (1977). A review of the distribution and biology of the potato cyst nematodes *Globodera rostochiensis* and *G. pallida*. *PANS* 23, 178-189.

FRANCO, J. (1979). Effect of temperature on hatching and multiplication of potato cyst nematodes. *Nematologica* 25, 237-244.

GRAINGER, J. (1964) Factors affecting the control of eelworm diseases. *Nematologica* 10, 5-20.

HOMINICK, W.M. (1986). Photoperiod and diapause in *Globodera rostochiensis*. *Nematologica* 32, 408-418.

HOMINICK, W.M. FORREST, J. M. & EVANS, A. A. F. (1985). Diapause in *Globodera rostochiensis* and variability in hatching trials. *Nematologica* 31, 159-170

JANSSEN, R., BAKKER, J. & GOMMERS, F. J. (1987) Circumventing the diapause of potato cyst nematodes. *Netherlands Journal of Plant Pathology* 93, 107-113.

JONES, F. G. W. (1970) The control of the potato cyst nematode. *Journal of the Royal Society of Arts*. 118, 170-199.

KERRY, B. R. & CRUMP, D. H. (1977). Observations on fungal parasites of females and eggs of the cereal cyst-nematode, *Heterodera avenae*, and other cyst nematodes. *Nematologica* 23, 193-201.

KLEYNHANS, K.P.N. (1998). Potato cyst nematodes (*Globodera* species) in Africa. In: Marks, R.J. & Brodie, B.B. (Eds.). *Potato Cyst Nematodes. Biology, Distribution and Control.* CAB International, Wallingford, Oxon OX10 8DE, UK, pp. 347 - 351.

LAMONDIA, J. A., RAWSTHORNE, D. & BRODIE, B. B. (1987). Decline of *Globodera rostochiensis* as influenced by Potato Root Diffusate movement and persistence in soil. *Journal of Nematology* 19, 172-176.

LE ROUX, S. (2000). Life cycle, epidemiology and control of the Golden cyst nematode (race: Ro1 under South African conditions at Ceres, Cape. *Report 1999-2000, Project No. 103402, ARC-Roodeplaat.*

MANKAU, R. (1980). Biological control of *Meloidogyne* populations by *Bacillus penetrans* in West Africa. *Journal of Nematology* 12, 230.

MARSHALL, J.W. (1998). Potato cyst nematodes (*Globodera* species) in New Zealand and Australia. In: Marks, R.J. & Brodie, B.B. (Eds.). *Potato Cyst Nematodes. Biology, Distribution and Control.* CAB International, Wallingford, Oxon OX10 8DE, UK, pp. 7 - 26.

MUHAMMAD, Z. & EVANS, A. A. F. (1997). Hatching in the potato cyst nematode *Globodera rostochiensis* indicating diapause as a cause of variability in emergence. *Nematologia Meditteranea* 25, 141-149.

MULDER, A. (1988b). Temperature response of *Globodera rostochiensis* and *G. pallida* (abstract). *Nematologica* 34, 281

MUNIR, A., PHILLIPS, M. S. & TRUDGILL, D. L. (2009). Effect of exposure to high temperatures on the hatching and reproduction of *Globodera rostochiensis*. *Pakistan Journal of Nematology* 27, 203-211

OOSTENBRINK, M. (1950). *Het Aardappelaaltje (*Heterodera rostochiensis *Wollenweber*), *een Gevaarlijke Parasiet voorde Eenzijdige Aardappel-cultuur*. Verslagen en Mededelingen van de Plantenziektenkundige Dienst no. 150, Wageningen, The Netherlands

ØYDVIN, J. (1978) *Studies on Potato Cyst nematodes*, Globodera *spp.* (*Skarbilovich) and the Use of Plant Resistance against* G. rostochiensis *(Woll) in Norway.* Växtskyddrapporter, Avhandlingar no. 2, Uppsala, Sweden, 37 pp.

PERRY, R. N. (1989) Dormancy and hatching of nematode eggs. *Parasitology Today* 5, 377-383.

PERRY, R. N. & BEANE, J. (1982) The effect of brief exposures to potato root diffusates on the hatching of *Globodera rostochiensis. Revue de Nématologie* 5, 221-224.

PRIDANNIKOV, M. V., PETELINA, G. G., PALCHUK, M. V., MASLER, E. P. & DZHAVAKHIYA, V. G. (2007). Influence of components of *Globodera rostochiensis* cysts on the *in vitro* hatch of second-stage juveniles. *Nematology* 9, 837-844.

ROBINSON, M. P., ATKINSON, H. J. & PERRY, R. N. (1987) The influence of temperature on the hatching, activity and lipid utilization of second stage juveniles of the potato cyst

nematodes *Globodera rostochiensis* and *G. pallida*. *Revue de Nématologie*; 10 (3), 349-354.

RYAN, N. A. & JONES, P. W. (2003). Effect of tuber-borne micro-organisms on hatching activity of potato root leachate towards potato cyst nematodes. *Nematology* 5, 55-63

SALAZAR, A. & RITTER, E. (1993). Effects of daylength during cyst formation, storage time and temperature of cysts on the in vitro hatching of *Globodera rostochiensis* and *G. pallida*. *Fundamental and Applied Nematology* 16, 567-572.

SCHLUTER, K. (1976) The potato cyst eelworm *Heterodera rostochiensis* Woll. in Morocco: its distrubution and economic importance. *Zeitschrift für Pflanzenkrankheiten und Pflanzenschutz* 83, 401-406.

SEINHORST, J. W. (1964). Methods for the extraction of *Heterodera* cysts from not previously dried soil samples. *Nematologica* 10, 87–94.

SHEPHERD, A.M. (1962). New blue R, a stain that differentiates between living and dead nematodes. *Nematologica* 8, 201-208.

STATSOFT, INC. (2012). STATISTICA (data analysis software system), version 11. www.statsoft.com.

STANTON, J. M. & SARTORI, M. (1990) Hatching and reproduction of the potato cyst nematode, *Globodera rostochiensis*, from potato fields in Western Australia as influenced by soil temperature. *Nematologica* 36: 457-464.

STIRLING, G. R. (1984). Biological control of *Meloidogyne javanica* with *Bacillus penetrans*. *Phytopathology* 74, 55-60.

TURNER, S. J. **(**1996). Population decline of potato cyst nematodes (*Globodera rostochiensis, G. pallida*) in field soils in Northern Ireland. *Annals of Applied Biology* 129, 315-322.

TURNER, S. J. & EVANS, K. (1998) The origins, global distribution and biology of potato cyst nematodes (*Globodera rostochiensis* (Woll.) and *Globodera pallida* Stone). In: Marks, R.J. & Brodie, B.B. (Eds.). *Potato Cyst Nematodes. Biology, Distribution and Control*. CAB International, Wallingford, Oxon OX10 8DE, UK, pp. 7 - 26.

TURNER, S. J., FLEMING, C. C., MORELAND, B. P. & MARTIN, T. J. G. (2009). Variation in hatch among pathotypes of the potato cyst nematodes, *Globodera rostochiensis* and *G. pallida*, in response to potato root diffusates from *Solanum* spp. I. Preliminary assessments to establish optimal testing conditions. *Nematology* 11(5), 749-756.

WHITEHEAD, A.G. (1992). Emergence of juvenile potato cyst-nematodes *Globodera rostochiensis* and *G. pallida* and the control of *G. pallida*. *Annals of Applied Biology* 120, 471-486.

WHITEHEAD, A. G. (1995). Decline of potato cyst nematodes, *Globodera rostochiensis* and *G. pallida*, in spring barley microplots. *Plant Pathology* 44, 191-195.

WINSLOW, R. D. & MCKENNA, L. A (1972). Comparative rates of development of strains of potato cyst nematode, *Heterodera rostochiensis*, in outdoor pot experiments. *Record of Agricultural Research, Ministry of Agriculture, Northern Ireland* 20, 17-20.

Table 9.1: Decline of populations of *Globodera rostochiensis* in infested plots monitored during this study.

Code	Farm	Region	GPS	Sampling date	Months from last crop	Cysts 100 cc^{-1}	Viable eggs 100 cc^{-1}
S	Nuwerus	Sandveld	S 32°19.233' EO 18°25.025'	20/10/09	0	7.33	181.67
				15/12/10	14	4.33	126.67
				19/01/12	27	4.40	80.67
				24/04/13	42	3.15	23.50
C	Alhambra	Ceres	S 33°22.001' EO 19°32.022'	17/02/09	0	3.33	136.67
				28/10/09	8	0.17	30.00
				17/02/11	24	0.60	22.00
				17/05/12	39	1.33	22.67
P	Nooitgedacht	South-Western Cape	S 34°02.086' EO 18°34.172'	02/11/10	36	27.83	0.00
H	Ripple Hill	Eastern Cape	S 33°45.993' EO 24°48.404'	13/07/10	36	57.67	590.00
				17/08/11	49	55.07	120.67
G	Chadinha bros	Gauteng	S 26°07.421' EO 27°34.856'	23/12/09	0	0.20	1.33
				13/05/11	17	0.00	0.00

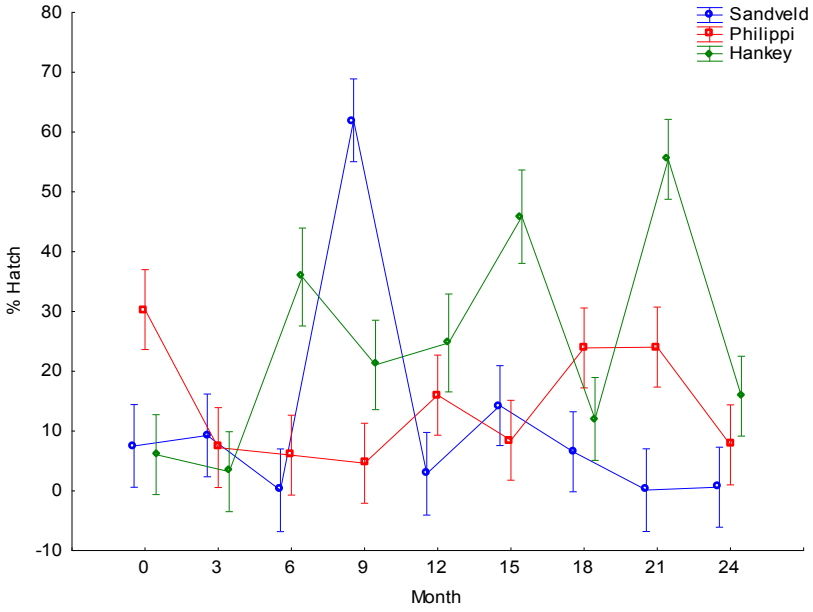

Fig. 9.1: Percentage hatch of juveniles of *Globodera rostochiensis* stored at 20°C for 3 – 24 months. Vertical bars denote 95% confidence intervals for the true means.

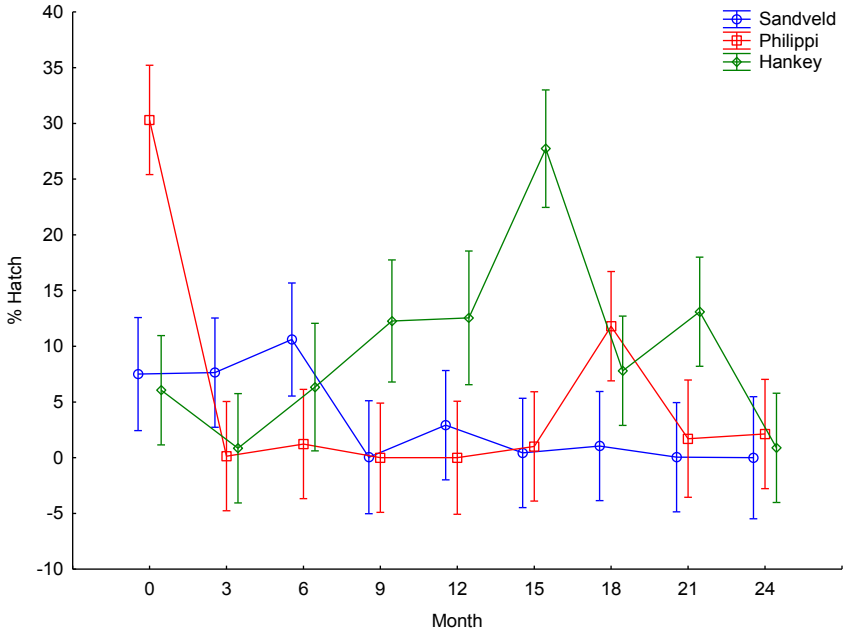

Fig. 9.2: Percentage hatch of juveniles of *Globodera rostochiensis* stored at 30°C for 3 – 24 months. Vertical bars denote 95% confidence intervals for the true means.

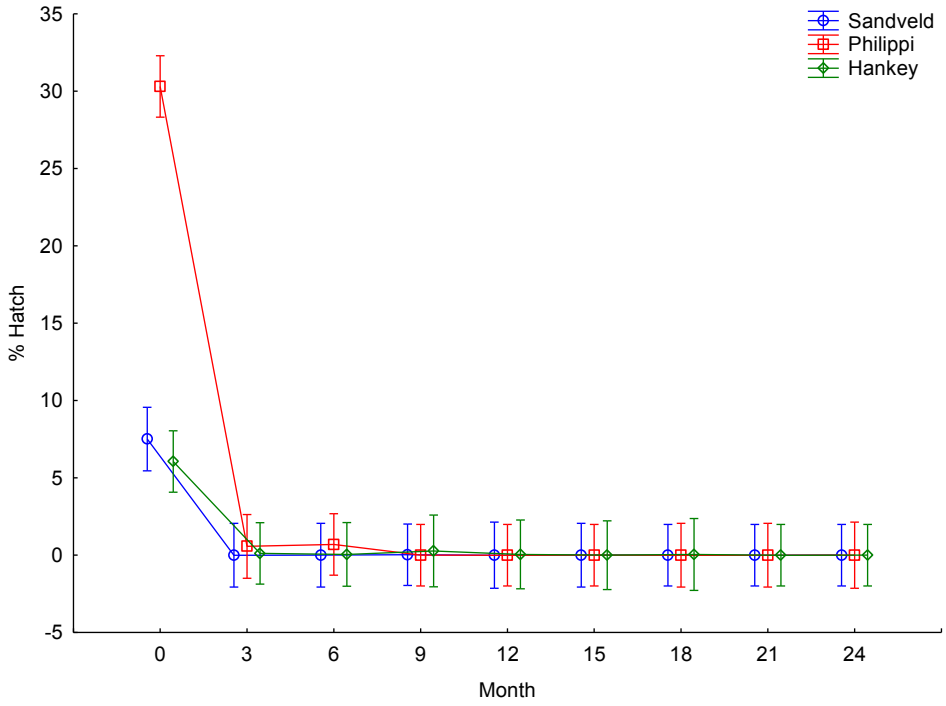

Fig. 9.3: Percentage hatch of juveniles of *Globodera rostochiensis* stored at 40°C for 3 – 24 months. Vertical bars denote 95% confidence intervals for the true means.

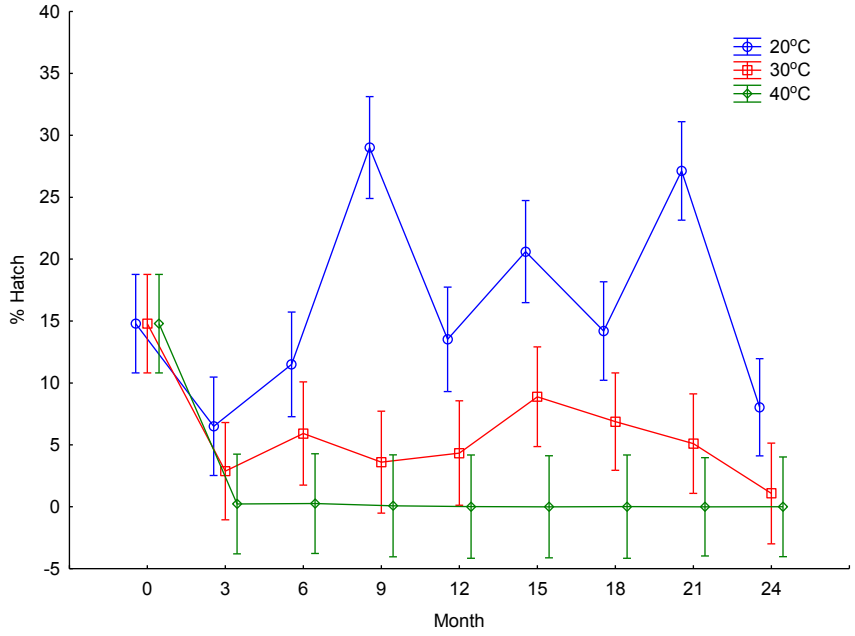

Fig. 9.4: Percentage hatch of juveniles of *Globodera rostochiensis* stored at different temperatures for 3 – 24 months. Vertical bars denote 95% confidence intervals for the true means.

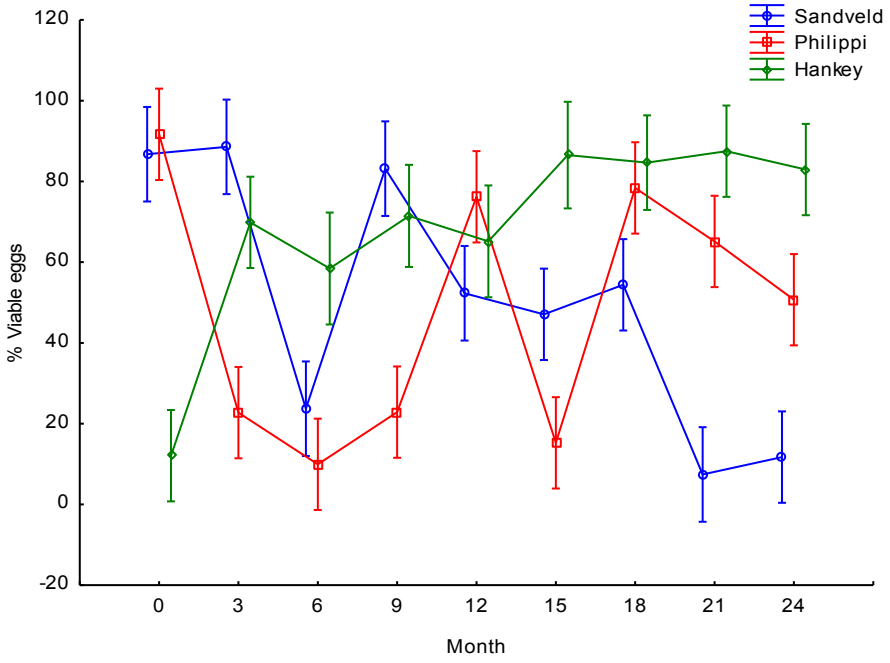

Fig. 9.5: Percentage of viable eggs per cyst of *Globodera rostochiensis* stored at at 20°C for 3 – 24 months. Vertical bars denote 95% confidence intervals for the true means.

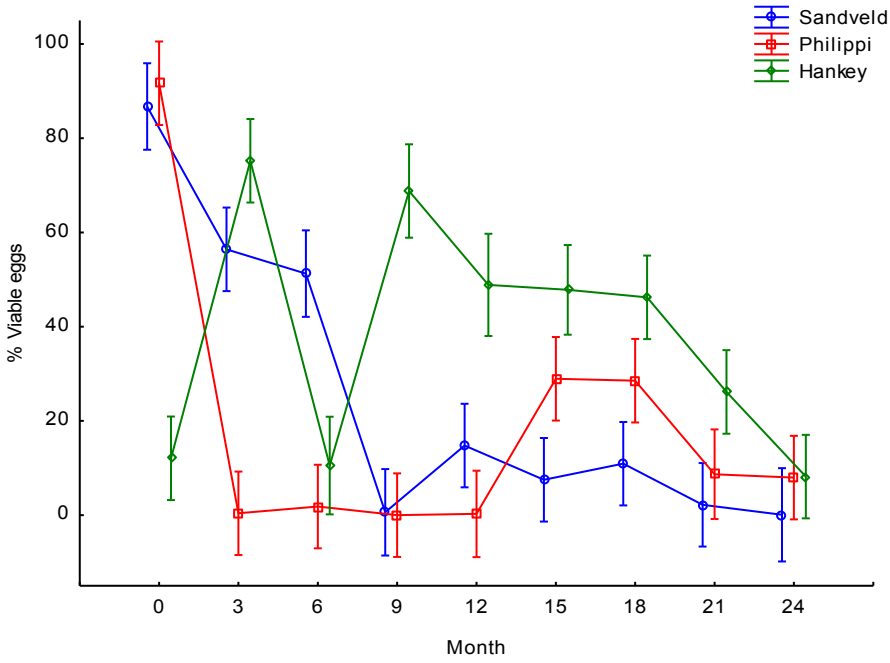

Fig. 9.6: Percentage of viable eggs per cyst of *Globodera rostochiensis* stored at at 30°C for 3 – 24 months. Vertical bars denote 95% confidence intervals for the true means.

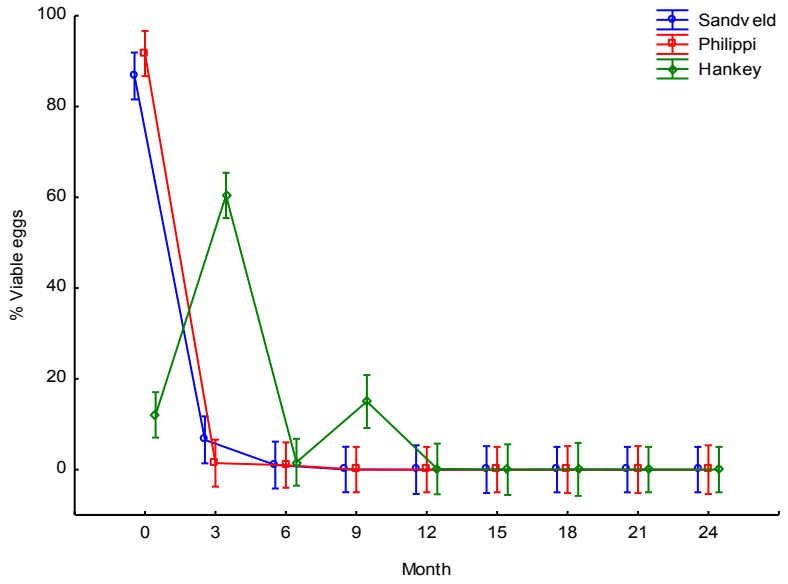

Fig. 9.7: Percentage of viable eggs per cyst of *Globodera rostochiensis* stored at at 40°C for 3 – 24 months. Vertical bars denote 95% confidence intervals for the true means.

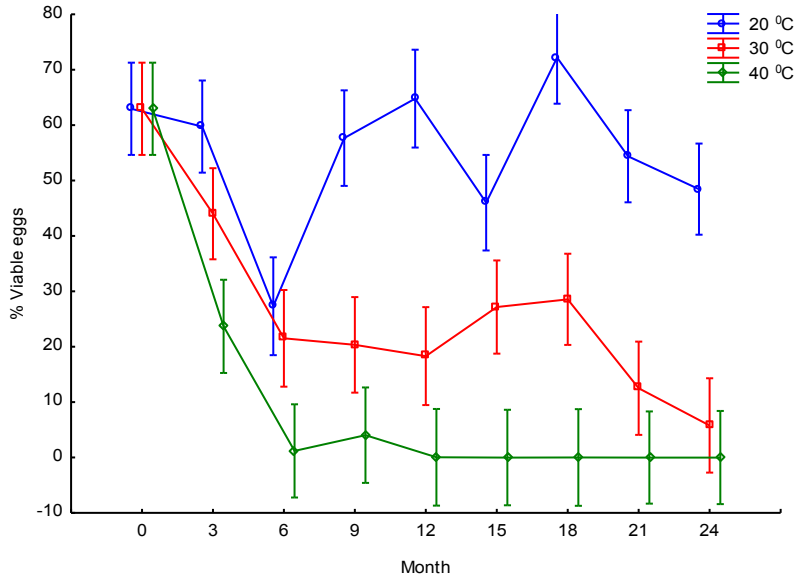

Fig. 9.8: Percentage of viable eggs per cyst of *Globodera rostochiensis* stored at different temperatures for 3 – 24 months. Vertical bars denote 95% confidence intervals for the true means.

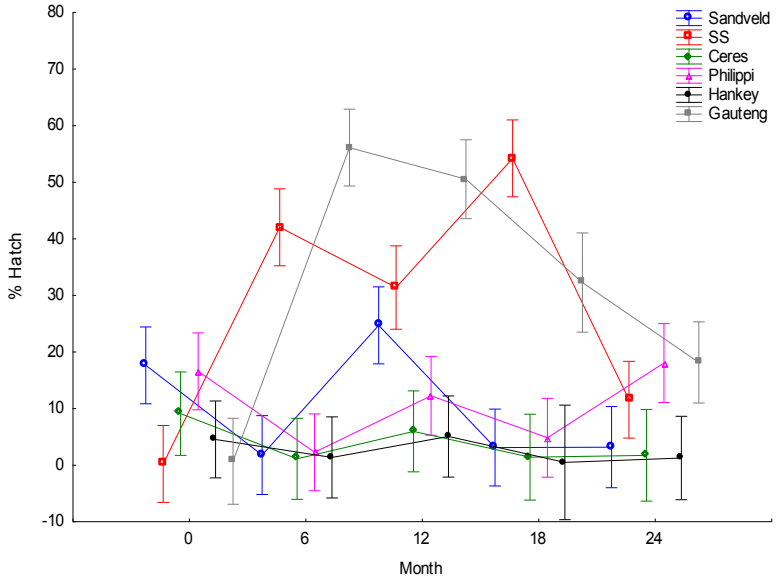

Fig. 9.9: Percentage hatch of juveniles of *Globodera rostochiensis* buried in field soil for 6 – 24 months. Vertical bars denote 95% confidence intervals for the true means.

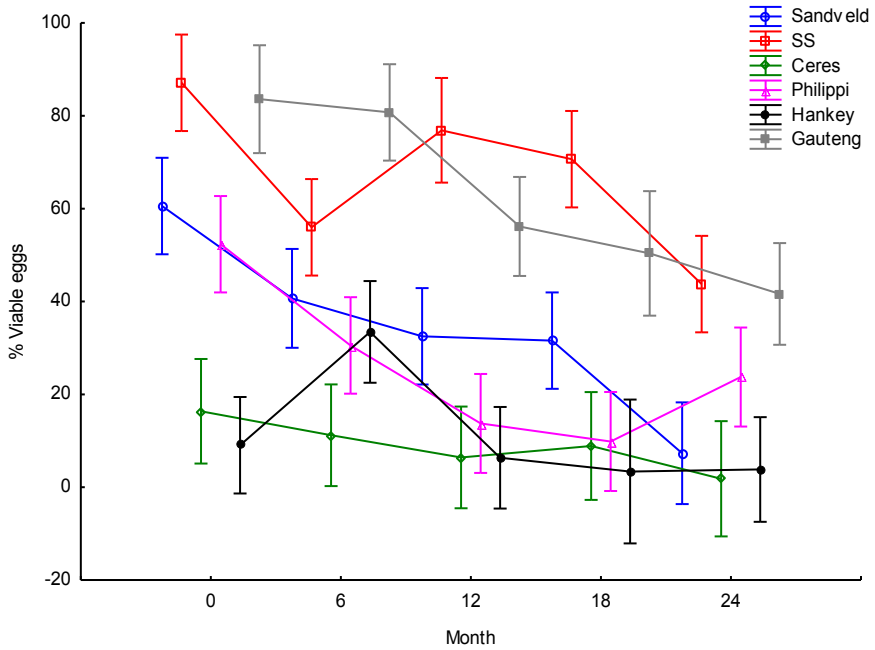

Fig. 9.10: Percentage of viable eggs per cyst of *Globodera rostochiensis* buried in field soil for 6 – 24 months. Vertical bars denote 95% confidence intervals for the true means.

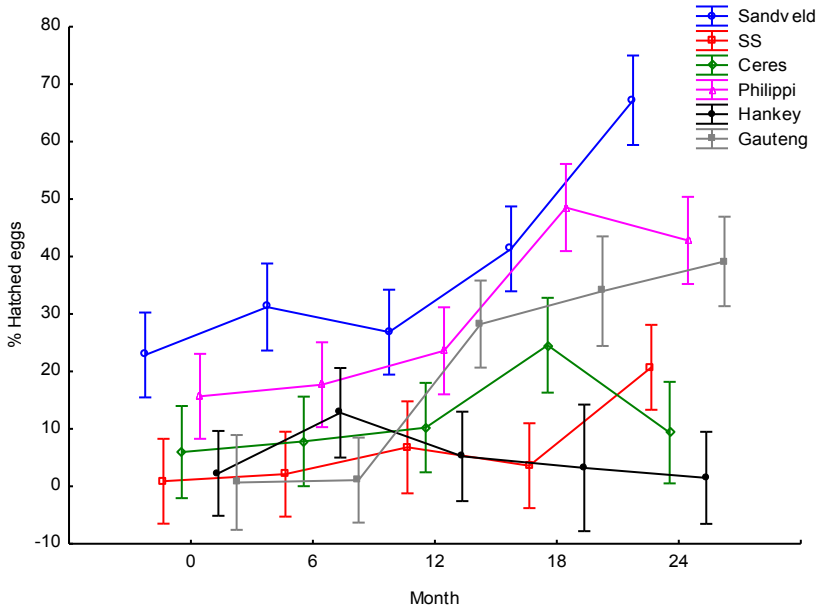

Fig. 9.11: Percentage of hatched eggs per cyst of *Globodera rostochiensis* buried in field soil for 6 – 24 months. Vertical bars denote 95% confidence intervals for the true means.

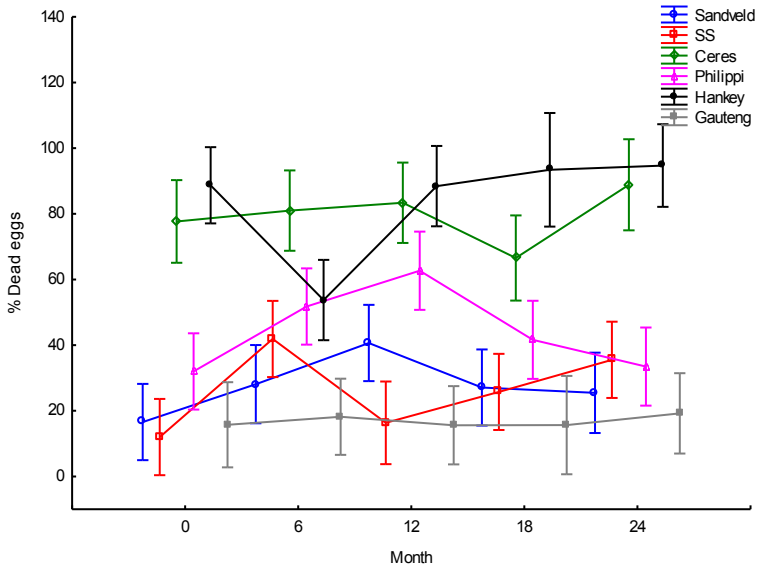

Fig. 9.12: Percentage of dead eggs per cyst of *Globodera rostochiensis* buried in field soil for 6 – 24 months. Vertical bars denote 95% confidence intervals for the true means.

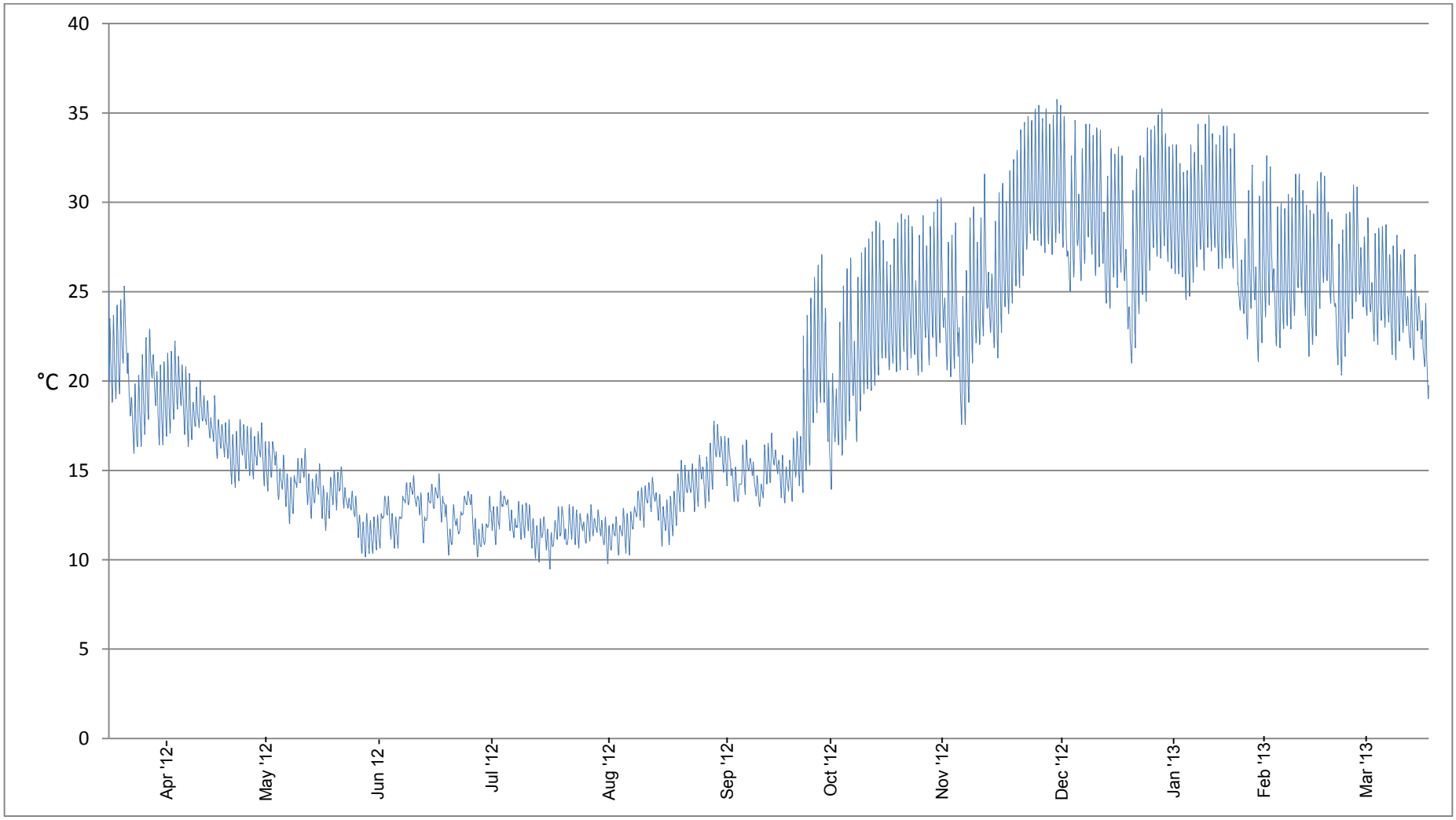

Fig. 9.13: Soil temperatures in the Stellenbosch location from April 2012 to March 2013.

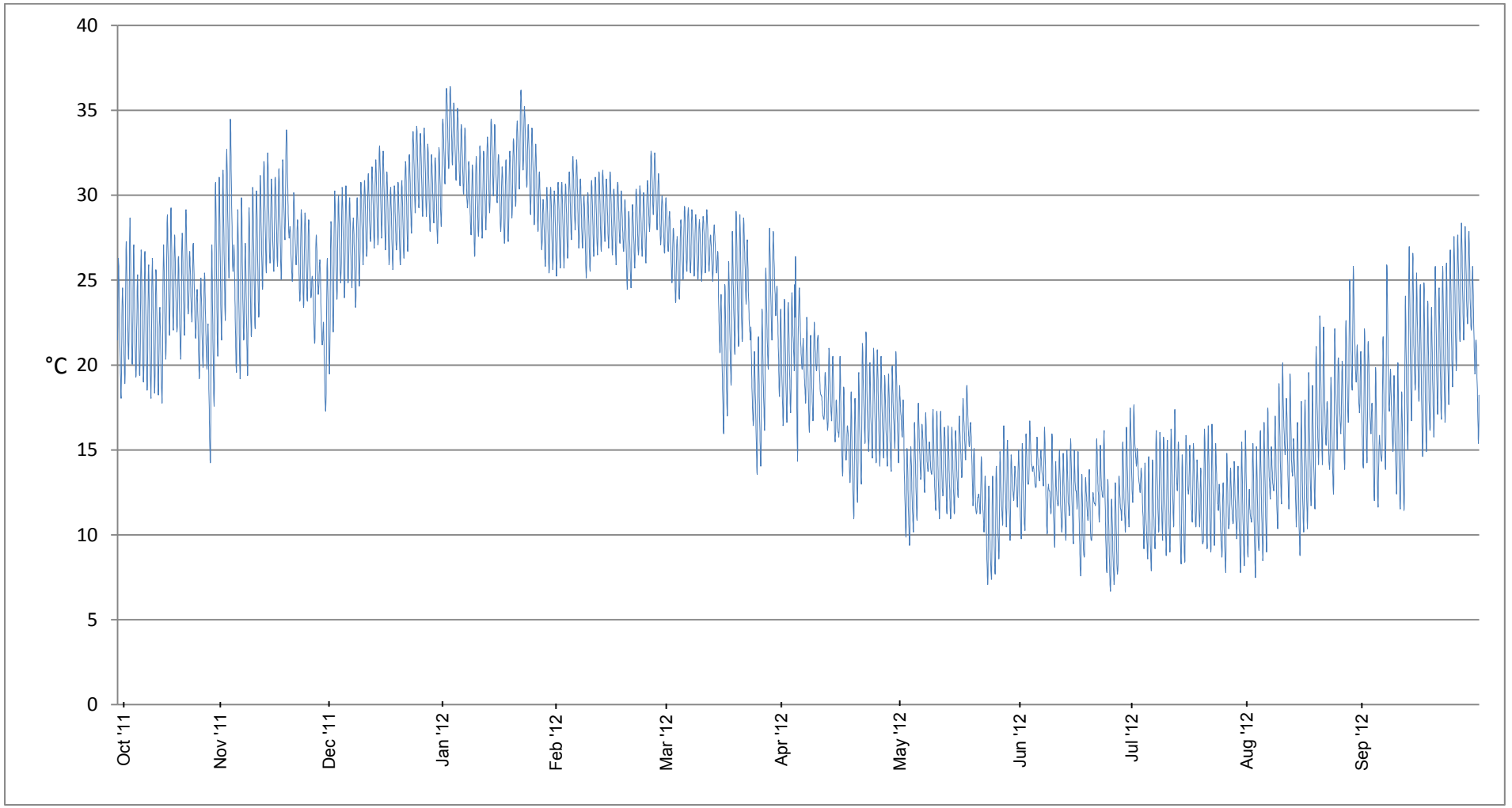

Fig. 9.14: Soil temperatures in the Sandveld location from October 2011 to September 2012.

Chapter 10

Assessing the *in vitro* reproduction ability of South African populations of *Globodera rostochiensis* (Nematoda: Heteroderidae)

Introduction

Some of the success of *Globodera rostochiensis* (Wollenweber, 1923) Skarbilovich, 1959 as an agricultural pest can be attributed to their adaptability to variable environments and their high reproduction potential (Turner & Evans, 1998). *G. rostochiensis* has been detected in some of the main potato-producing areas in South Africa, including the Sandveld, Ceres, Philippi, Hankey and Gauteng areas (Chapter 3). Severe climatic conditions occur in these regions, ranging from summer temperatures above 40°C in the Sandveld to winter temperatures below freezing in the Ceres area.

The life cycle of potato cyst nematodes comprises the egg, juveniles and adults. The active part of the life cycle starts when the second-stage juvenile (J2) hatches from the egg (Turner & Evans, 1998). The J2 is attracted to the growing root tip and enters the root by cutting through the cell walls with the stylet (Evans & Stone, 1977). Eventually it settles and begins feeding on cells in the pericycle, cortex or endodermis. Feeding induces enlargement of root cells and breakdown of cell walls to form a large syncytial ‘transfer cell’. The nematode remains feeding on the syncytium until its development is complete. At the third juvenile stage, females or males start to develop. Females become saccate and enlarge as her gonads increase in size. Males leave the root and are attracted to females that exude sex pheromones. After fertilisation, the embryos develop within the egg as far as the second juvenile stage

while still within the body of the female. The female then dies and transforms into a protective cyst containing 200-500 eggs (Turner & Evans, 1998).

In cool soils of northern Europe, there is usually one major generation of PCN each year (Jones, 1950); in moist soils, a partial second generation may occur but activity declines sharply at temperatures above 25°C (Berry *et al.*, 1977). The duration of the life cycle is dependent on planting date and is completed in about 90 days after invasion. In Chile, day degrees above 10°C required to reach the cyst stage were 358, 266 and 125 on potatoes planted in summer, spring and winter. Studies also showed that *G. rostochiensis* developed only one generation per growing season on spring potatoes in Chile. Although some emerged from J2 first-generation eggs, they were unable to reach the adult stage. In the same area 10%, 20% and 41% of the PCN reached the cyst stage at harvest of crops planted in summer, winter and spring. Populations increased 39-fold on spring potatoes but only eight- to nine fold on potatoes planted in summer or winter (Greco & Moreno, 1992a, b).

Multiplication rates of PCN on the host crop depend partly on the initial population density, because of competition for root space and its influence on sex ratio. With few eggs per gram of soil, multiplication may be 60-fold. With more than 100 eggs per gram soil, the postharvest population may be smaller than the initial population, because root systems become too severely damaged (Winslow & McKenna, 1972). The root form of the host plant also influences multiplication rates, with large root systems generally tolerating higher levels of invasion better than smaller ones and therefore permitting greater multiplication. In Long Island, New York 38-48 days are required for *G. rostochiensis* to develop from egg to adult (Chitwood & Buhrer, 1946). Depending on initial population density, reproduction of *G. rostochiensis* on Long Island is about 10 to 20-fold per growing season, with the greater increase at lower initial densities (Chitwood & Feldmesser, 1948).

Soil temperatures in excess of 26°C often exist on Long Island, which limits both reproduction and survival of *G. rostochiensis*. (Ferris, 1957). Population increase of *G. rostochiensis* in western New York ranges from 12 to 35-fold, with the greater increase at low initial population densities (LaMondia & Brodie, 1986). There was only one generation of *G. rostochiensis* per growing season in New York.

In Newfoundland, Canada, where soil temperatures are lower, the life cycle of *G. rostochiensis* requires 50-58 days to complete (Morris, 1971). There was only one generation of *G. rostochiensis* per growing season in Newfoundland. At Bon Accord, South Africa, the population density in one field increased from 2.1 eggs g^{-1} soil to only 4.2 eggs g^{-1} soil over 6 years of intermittent potato cropping (Kleynhans, 1988). Le Roux (2000) found that the life cycle of *G. rostochiensis* was completed within 6 weeks (42 days) after plant emergence (8 weeks after planting) under hot South African conditions. This is considerably less than the 90 days required under European conditions and the 80 days in New Zealand and Australia. Glasshouse studies also revealed that a small 2nd generation was possible within one potato season. In Italy a second generation of *G. rostochiensis* was started in some locations, but it was not completed (Greco *et al.*, 1988).

In New Zealand, *G. rostochiensis* passed a shorter period within the root (14 days) at 14°C than *G. pallida.* At 23°C the duration was shorter and equal for *G. rostochiensis* and *G. pallida* (9 days). *G. rostochiensis* completed the adult life stage more quickly (17.7 days) than *G. pallida*. The duration of the full life cycle shortened as soil temperature increased (Marshall, 1988). Stanton & Sartori (1990) found gravid females on roots between 35 and 49 days in Australia. The maximum number of eggs ml^{-1} soil produced in one generation was 182 and the maximum multiplication rate was 13 times (*Pi* 0.3 eggs ml^{-1} soil). The optimum temperature for the developing female and its egg was 15°C. Holz *et al.* (1999) found that

egg numbers per female varied with host for Bolivian potato cultivars that lack known partial resistance to *Globodera* spp. There was a 15-fold range between the most and least fecund nematode-host combinations. The effect of temperature on survival, fecundity and multiplication of three New Zealand populations was studied by Foot (1978). Temperature ranges for population increase of *G. rostochiensis* were 10 - 25°C. A study on the life history of *G. rostochiensis* in Cyprus showed that the nematode completes one generation per cropping season. The nematode also initiated, but failed to complete a second generation on the spring crop (Philis, 1980). Seinhorst (1992) illustrated consistent differences between numbers of eggs per cyst of pathotypes Ro 1 and Ro 3 of *G. rostochiensis* on a susceptible potato.

In this study the reproduction of different populations of *G. rostochiensis* at different temperatures will be examined.

Material and methods

OBTAINING CYSTS

Five geographic populations of *G. rostochiensis* from the Sandveld, Ceres, South-Western Cape, Eastern Cape and Gauteng potato-producing areas were evaluated. Cysts were obtained from potato tubers (cv. BP1) planted in washed sand in plastic pots with the drainage holes covered with milk filters. Soaked, viable cysts from the different populations were crushed and eggs and juveniles were added to the pots. The plants were maintained at a cycle of 14h light and 10h dark at 25°C in a glasshouse. After 12 weeks, new cysts were extracted using a Seinhorst cyst elutriator (Seinhorst, 1964). Cysts were stored in plastic vials in the dark at

20°C for one year after harvesting to overcome diapause before using them in these experiments.

REPRODUCTIVE TESTS

Germinating potato tubers (cv. BP1) were disinfected with 5% household bleach for 2 min, rinsed with water and dried. The tubers were placed in a closed container with 200 g dried clean sand and 30 ml distilled water. The tubers were incubated in the dark at 20°C, allowing roots to develop. Cysts were first soaked in potato root diffusates (PRD) for 3 days at 20°C. Inoculum was prepared by crushing in a microcentrifuge tube with a homogeniser. The eggs and juvenile contents were released from the cysts by vortexing. The eggs were separated from cyst fragments by washing them through a 90 µm sieve, collecting on a 25 µm sieve. Eggs caught on the 25 µm sieve were transferred to a beaker and the amount of eggs ml^{-1} was estimated by removing two aliquots and counting in a counting chamber. The containers with the potato were then inoculated with approximately 1000 eggs by pipetting the egg suspension directly into the sand. The tubers were then incubated in the dark at 15°C, 20°C and 25°C (5 repetitions each) for 12 weeks.

After 12 weeks the cysts and females were extracted with a Seinhorst cyst elutriator (Seinhorst, 1964). For each container the total number of cysts was counted and the numbers of eggs estimated by counting an aliquot of the eggs removed from the cysts as before. Estimates of survival (number of cysts / number of eggs added originally), fecundity (eggs / new cyst) and multiplication (number of new eggs / number of eggs added originally) were made.

DATA ANALYSIS

Data were analysed either with a factorial analysis of variance (ANOVA) if more than one factor was involved or with one-way ANOVA if only one factor was involved. In all tests a significance level of 5% was used. If significant interaction was observed in the factorial ANOVA, the interaction effects/means were analysed with Bonferroni multiple comparisons to detect which interaction effects differ. If the interaction was not significant, the main effects could be interpreted and again Bonferroni multiple comparisons were used to detect differences among the main effects/means. All statistical analyses were done with STATISTICA version 11 (StatSoft Inc. 2012).

Results

Data analysed with a factorial ANOVA showed significant differences ($F_{(8, 56)} = 3.1007$; $P = 0.00574$) when comparing the survival (number of cysts / number of eggs added) of the populations at different temperatures. The populations responded similarly in terms of survival, except for the Philippi population, where the highest survival rate was at 15°C (0.12 ± 0.04), whereas the highest survival rate for all the other populations was at 20°C. For all populations the lowest survival rate was at 25°C (Figure 10.1). The rate of survival did however not differ significantly ($P > 0.05$) at the different temperatures for the Sandveld and Gauteng populations. The survival rate of the Ceres population was significantly lower at 25°C and 15°C ($P < 0.05$) than the survival rate at 20°C. The survival rate of the Philippi

population at 15°C was significantly higher than the survival rate at 25°C for this population. The survival rate Hankey population was significantly lower at 25°C than at 20°C.

Data analysed with a factorial ANOVA did not show significant differences ($F_{(8, 50)}$ = 1.8040; *P* = 0.09844) when comparing the fecundity (eggs / new cyst) of the populations at different temperatures (Figure 10.2). However when the main effects were interpreted again, it showed that the fecundity of cysts did differ significantly at the different temperatures ($P < 0.05$). The highest fecundity was at 15°C for all populations (197 ± 48).

Data analysed with a factorial ANOVA showed significant differences ($F_{(8, 56)}$ = 2.3163; *P* = 0.03189) when comparing the multiplication (number of new eggs / number of eggs added originally) of the populations at different temperatures. The highest multiplication (number of new eggs / number of eggs added originally) for S, C and H observed at 20°C, but for P and G the highest multiplication rate was at 15°C (Figure 10.3). The lowest multiplication rates were observed at 25°C (1.4 ± 3.3). Only the multiplication rate of the Gauteng population was above 1 (6.5 ± 5.3) at this temperature. The only significant differences observed was with the Ceres and Hankey populations were the multiplication rate at 25°C was significantly lower than at 20°C and the Gauteng population were the multiplication rate at 25°C was significantly lower than at 15°C. The higher temperature at which the multiplication rate drops below 5 times ranges between 23°C and 25°C for all the populations.

Discussion

The results of these experiments confirm that the optimum temperature for the reproduction of *Globodera* is around 20°C, but subtle differences were observed between the populations.

Data suggests that the Philippi population could be more adapted for survival and multiplication at temperatures lower than 20°C than the other populations, whereas the Gauteng population was the only population that could maintain a multiplication rate higher than 1 at 25°C. The data suggests that for all the populations, multiplication and survival is influenced significantly when the temperatures rise above 25°C. This would mean that the reproduction of these nematodes in warm soils would be significantly hampered. As shown in Chapter 10, the average temperature of soils in two measured "hot" regions is around 20°C, which suggests that the reproduction of *G. rostochiensis* will not be negatively influenced by temperature in these regions. Crop losses (above 50%) from infested potato fields in the Ceres production area (Le Roux, 2000) are believed to be an indication that the soil temperatures in these regions do not influence the reproduction of *G. rostochiensis* negatively.

Literature

BERRY, M. M., STONE, A. R. & PARROTT, D. M. (1977). Nematology Department. In: *Report of Rothamsted Experimental Station for 1976, Part 1*, p198.

CHITWOOD, G.B. & BUHRER, E.M. (1946). The life history of the golden nematode of potatoes, *Heterodera rostochiensis* Wollenweber, under Long Island, New York conditions. *Phytopathology* 36, 180-189.

CHITWOOD, G.B. & FELDMESSER, J. (1948). Golden nematode population studies. *Helminthological Society of Washington Proceedings* 15, 43-55.

EVANS, K. & STONE, A. R. (1977). A review of the distribution and biology of the potato cyst nematodes *Globodera rostochiensis* and *G. pallida*. *PANS* 23, 178-189.

FOOT, M.A. (1978). Temperature responses of three potato cyst nematode populations from New Zealand. *Nematologica* 24, 412-417.

GRECO, N., INSERRA, R. N., BRANDONISIO, A., TIRRO, A. & MARINIS, G. DE. (1988). Life cycle of *Globodera rostochiensis* on potato in Italy. *Nematologica Meditterranea* 16, 69-73.

GRECO, N. & MORENO, I. (1992a) Influence of *Globodera rostochiensis* on yield of summer, winter and spring potato in Chile. *Nematrópica* 22, 165-173.

GRECO, N. & MORENO, I. (1992b) Development of *Globodera rostochiensis* during three different growing seasons in Chile. *Nematropica* 22, 175-181.

HOLZ, R. A., TROTH, K. & ATKINSON, H. J. (1999). The Influence of Potato Cultivar on Lipid Content and Fecundity of Bolivian and British Populations of *Globodera rostochiensis*. *Journal of Nematology* 31(4):357–366.

JONES, F. G. W. (1950). Observations on the beet eelworm and other cyst-forming species of *Heterodera*. *Annals of Applied Biology* 37, 407-440.

KLEYNHANS, K.P.N. (1998). Potato cyst nematodes (*Globodera* species) in Africa. In: Marks, R.J. & Brodie, B.B. (Eds.). *Potato Cyst Nematodes. Biology, distribution and control.* CAB International, Wallingford, Oxon OX10 8DE, UK, pp. 347 - 351.

LAMONDIA, J. A. & BRODIE, B. B. (1986). Effects of initial nematode density on population dynamics of *Globodera rostochiensis* on resistant and susceptible potatoes. *Journal of Nematology* 18 (2), 159-165.

LE ROUX, S. (2000). Life cycle, epidemiology and control of the Golden cyst nematode (race: Ro1 under South African conditions at Ceres, Cape. *Report 1999-2000, Project No. 103402, ARC-Roodeplaat.*

MARSHALL, J.W. (1998). Potato cyst nematodes (*Globodera* species) in New Zealand and Australia. In: Marks, R.J. & Brodie, B.B. (Eds.). *Potato Cyst Nematodes. Biology, Distribution and Control.* CAB International, Wallingford, Oxon OX10 8DE, UK, pp. 7 - 26.

MORRIS, R.F. (1971). Distribution and biology of the golden nematode, *Heterodera rostochiensis* in Newfoundland. *Nematologica* 17, 370-376.

PHILIS, J. (1980). Life history of the potato cyst nematode *Globodera rostochiensis* in Cyprys. *Nematologica* 26, 295-301.

SEINHORST, J. W. (1964). Methods for the extraction of *Heterodera* cysts from not previously dried soil samples. *Nematologica* 10, 87–94.

SEINHORST, J.W. (1992) The relation between rate of reproduction and average number of eggs per cyst in *Globodera rostochiensis* and *G. pallida. Nematologica* 38, 335-352.

STANTON, J.M. & SARTORI, M. (1990) Hatching and reproduction of the potato cyst nematode, *Globodera rostochiensis*, from potato fields in Western Australia as influenced by soil temperature. *Nematologica* 36: 457-464.

STATSOFT, INC. (2012). STATISTICA (data analysis software system), version 11. www.statsoft.com.

TURNER, S.J. & EVANS, K. (1998) The origins, global distribution and biology of potato cyst nematodes (*Globodera rostochiensis* (Woll.) and *Globodera pallida* Stone). In: Marks, R.J. & Brodie, B.B. (Eds.). *Potato Cyst Nematodes. Biology, Distribution and Control*. CAB International, Wallingford, Oxon OX10 8DE, UK, pp. 7-26.

WINSLOW, R.D. & MCKENNA, L.A (1972). Comparative rates of development of strains of potato cyst nematode, *Heterodera rostochiensis*, in outdoor pot experiments. *Record of Agricultural Research, Ministry of Agriculture, Northern Ireland* 20: 17-20.

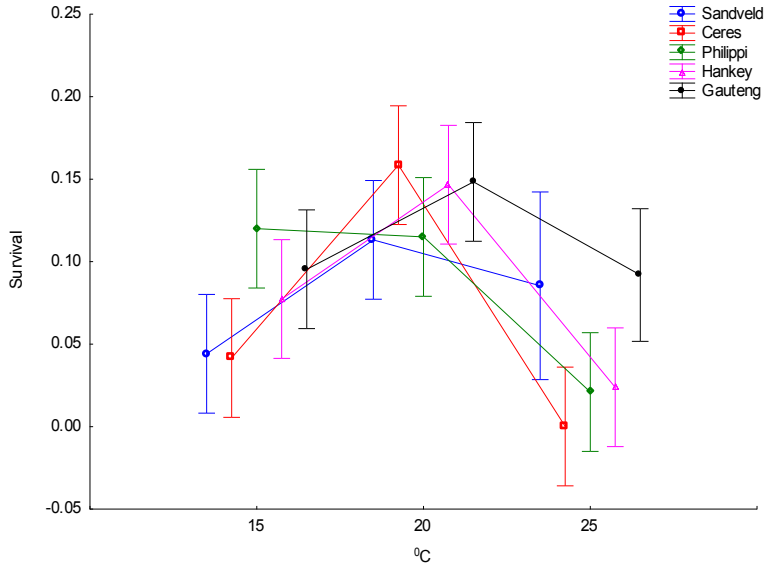

Fig. 10.1: Survival (number of cysts / number of eggs added originally) of five populations of *G. rostochiensis* at 15°C, 20°C and 25°C.

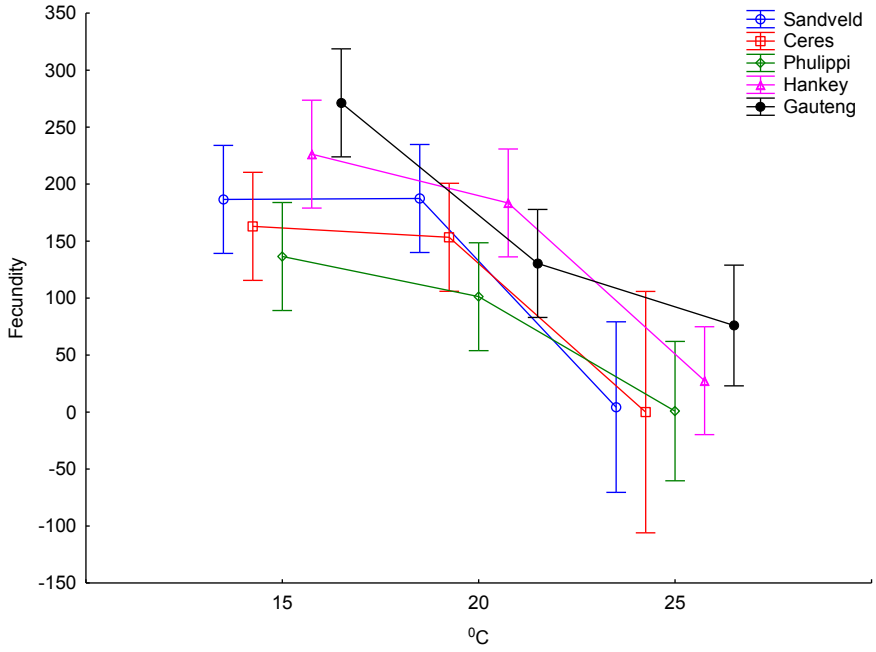

Fig. 10.2: Fecundity (eggs / new cyst) of five populations of *G. rostochiensis* at 15° C, 20°C and 25°C.

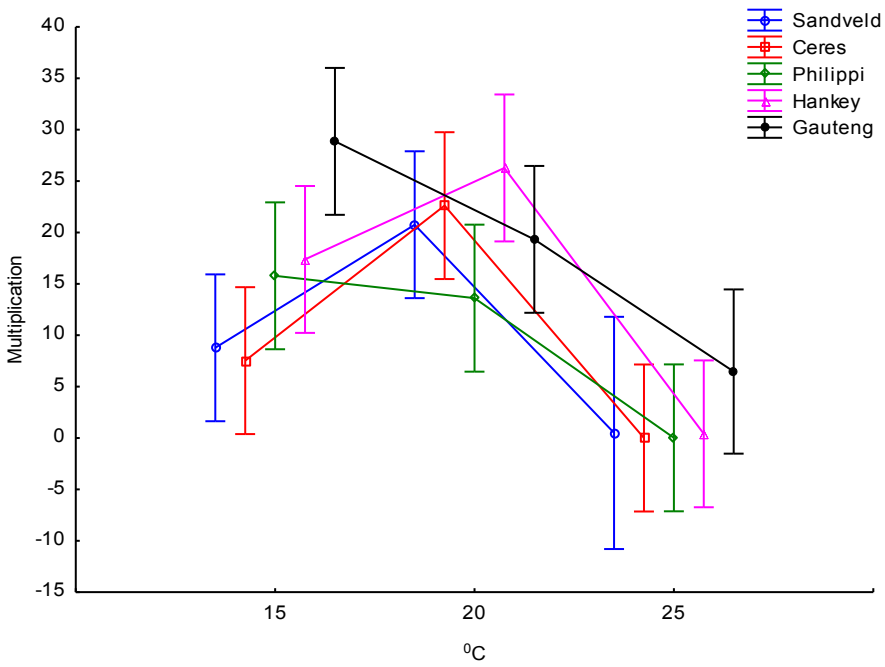

Fig. 10.3: Multiplication (number of new eggs / number of eggs added originally) of five populations of *G. rostochiensis* at 15° C, 20°C and 25°C.

Chapter 11

Determining the pathotypes of South African populations of *Globodera rostochiensis* (Nematoda: Heteroderidae)

Introduction

Coevolution between potato cyst nematodes (PCN) and *Solanum* species has led to the development of species differences, as well as intraspecific diversity, within both *Globodera rostochiensis* (Wollenweber, 1923) Skarbilovich, 1959 and *Globodera pallida* Stone, 1973 (Fleming & Powers, 1998). This diversity was initially observed when the reproductive ability of PCN populations was tested on *Solanum* clones containing PCN resistance genes. Many PCN populations could reproduce on *Solanum* clones despite the presence of these resistance genes. This led to the recognition of a series of virulent 'resistance-breaking' pathotypes within both PCN species (Fleming & Powers, 1998). Knowledge of the genetic variability and area of origin of PCN populations should be taken into account when developing resistant varieties (Grenier *et al.*, 2010). The distinction since 1972 between *G. pallida* and *G. rostochiensis* has revealed that numerous resistance genes against one species are not efficient against the other (Bakker *et al.*, 1993). Peruvian *Solanum* species are generally resistant to *G. pallida* whilst those from Bolivia and Argentina generally combine both *G. rostochiensis* and *G. pallida* resistance (Turner, 1989).

Attempts were made in South America and Europe to develop formalized schemes for PCN pathotype nomenclature and characterisation. Two international schemes were proposed, both differentiating between PCN pathotypes on the basis of their multiplication rates on a series

of differential potato clones (Canto Saenz & de Scurrah, 1977; Kort *et al.*, 1977). These schemes initially identified up to five *G. rostochiensis* and six *G. pallida* pathotypes. However, inherent limitations and problems became apparent when using these schemes, particularly when attempting to characterize PCN field populations that contain species or pathotype mixtures (Stone, 1985; Trudgill, 1985). Trudgill (1985) argued that, within the European scheme, only pathotypes Rol, Ro4 and Pal could give unequivocal responses and thus be regarded as true pathotypes. More recently, Nijboer and Parlevliet (1990) proposed that within *G. rostochiensis*, only pathotypes Rol (old Rol and Ro4), Ro3 (old Ro2 and Ro3) and Ro5 should be recognized. Therefore Ro1 is distinguishable by reproduction on susceptible cultivars but not on those containing the H1 gene, while Ro3 will reproduce on both. Ro5 will reproduce on both, but also on the clone *S. vernei*, 58.1642.4.

Stone *et al.* (1986) found only the Ro1 pathotype of *G. rostochiensis* in a survey of potato cyst nematode pathotypes in Great Britain, using the method of Kort *et al.* (1977). In Spain, 31 *G. rostochiensis* populations were identified as pathotype Ro1 by Martinez-Beringola *et al.* (1987). None reproduced on clones derived from *Solanum andigena.* Seinhorst (1992) illustrated consistent differences between numbers of eggs per cyst of pathotypes Ro1 and Ro3 of *G. rostochiensis* on a susceptible potato. Zaheer *et al.* (1993) tested 78 populations of *G. rostochiensis* in Northern Ireland to determine the presence of gene H1 resistance-breaking populations. In these tests all populations failed to increase on Maris Piper, indicating that only Rol was present. Bendezu *et al.* (1998) reared populations of *G. rostochiensis* and *G. pallida* from Europe and Bolivia on 3 commercial potato cultivars and 5 differential potato clones to show differences in virulence between populations. The virulence bioassay distinguished between species but failed to classify the populations into pathotypes. Hinch *et al.* (1998) used high performance capillary electrophoresis to differentiate between

pathotypes of *G. rostochiensis* and *G. pallida*. Australian isolates from Victoria was found to be Ro1. This pathotype classification was also confirmed by exposing each population to a fully susceptible control cultivar, a cultivar containing the resistance gene H1 or a potato line containing the resistance gene H2. Greco *et al.* (2007) investigated the pathotypes and heterogeneity of Italian populations of *G. rostochiensis*. All populations of *G. rostochiensis* reproduced on a susceptible potato cultivar and on one containing the H1 gene, indicating it to be Ro3. Hlaoua *et al*. (2008) proved that *G. rostochiensis* populations in Tunisia belong to pathotype Ro1 by inoculating susceptible and resistant cultivars (carrying the H1 gene) in petri dishes and observing the development of females on the roots. Only Ro1 pathotypes are not able to develop females on resistant pathotypes.

Le Roux (2000) refers to the population of *G. rostochiensis* in Ceres as pathotype Ro1. The classification was done by Dr. Colin Fleming (Applied Plant Science, Agri-Food and Bioscience Institute, Newforge Lane, Belfast BT9 5PX, United Kingdom) by reproduction on a set of differential potato clones (Susceptible cultivar Desiree and resistant cultivar Maris Piper).

To enable growers to make informed decisions w.r.t. selection of cultivars for the management of these nematodes, it is imperative that the pathotype of the South African populations are established.

Materials and methods

OBTAINING CYSTS

Five geographic populations of *G. rostochiensis* were evaluated in this study. They originate from unique locations in the Sandveld, Ceres, South-Western Cape, Eastern Cape and Gauteng potato-producing areas. Cysts used in this experiment were obtained from cultures. Germinating potato tubers (cv. BP1) were disinfected with 5% household bleach for 2 minutes rinsed with water and dried. The tubers were placed in a closed container with 200 g dried sand and 30 ml distilled water. The tubers were incubated in the dark at 20°C, allowing roots to develop. Soaked, viable cysts from the different populations were crushed and eggs and juveniles were added to the containers. The tubers were then incubated in the dark at 20°C. After 12 weeks new cysts were extracted using a Seinhorst cyst elutriator (Seinhorst, 1964). Cysts obtained in this manner were stored in plastic vials in the dark at 20°C for one year after harvesting to overcome diapause before using them in these experiments.

REPRODUCTIVE TESTS

Germinating potato tubers, one susceptible cultivar (cv. BP1) and one resistant cultivar containing the H1 gene (cv. Mondial) were disinfected with 5% household bleach for 2 minutes, rinsed with water and dried. The tubers were placed in a closed container with 200 g dried, clean sand and 30 ml distilled water. The tubers were incubated in the dark at 20°C, allowing roots to develop.

Cysts were first soaked in potato root exudates (PRD) for 3 days at 20°C. PRD was obtained by removing one month old potato plants (cv. BP1) from the pot and rinsing the roots to

remove soil particles. The plants were then kept at 25°C with the roots in 50 ml distilled water in glass beakers for 24 h. The diffusates were then passed through a 90 µm sieve and stored at 4°C. Before use, the diffusate was sterilised by passing through a 0.45 µm pore cellulose-acetate filter.

Inoculum was prepared by crushing cysts in an Eppendorf tube with a homogeniser. The eggs and juvenile contents were released from the cysts by vortexing. The eggs were separated from cyst fragments by washing them through a 90 µm sieve, collecting on a 25 µm sieve. Eggs caught on the 25 µm-pore sieve were transferred to a beaker and the amount of eggs ml^{-1} was estimated by removing two aliquots and counting in a counting chamber. The containers with the potato were then inoculated with approximately 1000 eggs by pipetting the egg suspension directly into the sand. The tubers were then incubated in the dark at 20°C (5 repetitions each) for 12 weeks and the cysts and females were extracted with a Seinhorst cyst elutriator (Seinhorst, 1964). For each container the total number of cysts was counted and the numbers of eggs estimated by counting an aliquot of the eggs removed from the cysts as before. Estimates of survival (number of cysts / number of eggs added originally) and multiplication (number of new eggs / number of eggs added originally) were made.

Results

The results of the reproductive test are shown in Table 11.1. Multiplication rates for all the populations on susceptible cultivars were higher than 1 (Average = 16.4). Multiplication rates for all the populations on the resistant cultivar were lower than 1 (Average = 0.0002). Survival rates on the resistant cultivar were negligible.

Discussion

The populations tested clearly all belong to pathotype Ro1 (old Rol and Ro4), showing no multiplication on a resistant cultivar containing the H1 gene. There was no need for testing with clone *S. vernei*, 58.1642.4, because pathotype Ro5 would also have reproduced on the resistant cultivar Mondial. In Chapter 5, we concluded that the Sandveld population was genetically different from the other populations of *G. rostochiensis* tested. The genetical variation between the populations was in this case not indicative of the occurrence of different pathotypes.

Literature

BAKKER, J., FOLKERTSMA, R.T., ROUPPE VAN DER VOORT, J.N., DE BOER, J.M. & GOMMERS, F.J. (1993). Changing concepts and molecular approaches in the management of virulence genes in potato cyst nematodes. *Annual Review of Phytopathology* 31, 169-190.

BENDEZU, I. F., RUSSELL, M.D. & EVANS, K. (1998). Virulence of populations of potato cyst nematodes (*Globodera* spp.) from Europe and Bolivia towards differential potato clones frequently used for pathotype classification. *Nematologica* 44(6), 667-681.

CANTO SAENZ, M. & DE SCURRAH, M.M. (1977). Races of the potato cyst nematode in the Andean region and a new system of classification. *Nematologica* 23, 340-349.

FLEMING, C. C. & POWERS, T. O. (1998). Potato cyst nematodes: species, pathotypes and virulence concepts. In: Marks, R.J. & Brodie, B.B. (Eds.). *Potato Cyst Nematodes. Biology, distribution and control.* CAB International, Wallingford, Oxon OX10 8DE, UK, pp. 51 - 57.

GRECO, N., BRANDONISIO, A. & COSMIS, P. DE (2007). Pathotypes and heterogeneity of Italian populations of *Globodera rostochiensis* and *G. pallida*. *Nematologia Mediterranea* 35, 137-142.

GRENIER, E., FOURNET, S., PETIT, E. & ANTHOINE, G. (2010). A cyst nematode 'species factory' called the Andes. *Nematology* 12, 163-169.

HINCH, J.M., ALBEDI, F., SMITH S. C., WOODWARD, J. R. & EVANS, K. (1998). Discrimination of European and Australian *Globodera rostochiensis* and *G. pallida* pathotypes by high performance capillary electrophoresis. *Fundamental and Applied Nematology* 21, 123-128.

HLAOUA, W., HORRIGUE-RAOUANI, N., FOUVILLE, D. & MUGNIERY, D. (2008) Morphological and molecular characterisation of potato cyst nematode populations from Tunisia and survey of their geographical origin. *Biotechnology* 7, 651-659.

KORT, J., ROSS, H., RUMPENHORST, H. J. & STONE, A.R. (1977). An international scheme for identifying and classifying pathotypes of potato cyst nematodes *Globodera rostochiensis* and *G. pallida*. *Nematologica* 23, 333-339.

LE ROUX, S. (2000). Life cycle, epidemiology and control of the Golden cyst nematode (race: Ro1) under South African conditions at Ceres, Cape. *Report 1999-2000, Project No. 103402, ARC-Roodeplaat.*

MARTINEZ-BERINGOLA, M. L., FRANCO, L., PAZ-VIVAS, L. M. & GUTIERREZ, M. P. (1987) Spanish pathotypes of *Globodera rostochiensis* and *G. pallida*. *Nematologia Mediterranea* 15, 193-203.

NIJBOER, H. & PARLEVLIET, J.E. (1990). Pathotype-specificity in potato cyst nematodes, a reconsideration. *Euphytica* 49, 39-47.

SEINHORST, J.W. (1964). Methods for the extraction of *Heterodera* cysts from not previously dried soil samples. *Nematologica* 10, 87–94.

SEINHORST, J. W. (1992) The relation between rate of reproduction and average number of eggs per cyst in *Globodera rostochiensis* and *G. pallida*. *Nematologica* 38, 335-352.

STONE, A.R. (1985). Co-evolution of potato cyst nematodes and their hosts: implications for pathotypes and resistance. *EPPO Bulletin* 15, 131-137.

STONE, A. R., HOLLIDAY J. M., MATHIAS, P. L. & PARROTT, D. M. (1986). A selective survey of potato cyst-nematode pathotypes in Great Britain. *Plant Pathology* 35, 18-24

TRUDGILL, D.L. (1985) Potato cyst nematodes: a critical review of the current pathotyping scheme. *EPPO Bulletin* 15, 273-279.

TURNER, S.J. (1989). New sources of resistance to potato cyst-nematodes in the Commonwealth Potato Collection. *Euphytica* 42, 145-153.

ZAHEER, K., FLEMING, C. C. & TURNER, S. J. (1993). Distribution and frequency of occurrence of potato cyst nematode pathotypes in Northern Ireland. *Plant Pathology* 42, 609-616.

Table 11.1: Reproduction of different populations of *Globodera rostochiensis* on susceptible and resistant potato cultivars.

Cyst population	Potato cultivar	Survival	Multiplication
Sandveld	BP1	0.109	12.295
	Mondial	0.000	0
Ceres	BP1	0.149	15.548
	Mondial	0.000	0.0201
South-western Cape	BP1	0.133	22.989
	Mondial	0.000	0.000
Eastern Cape	BP1	0.0263	2.373
	Mondial	0.001	0.200
Gauteng	BP1	0.140	28.736
	Mondial	0.000	0.000

Chapter 12

Reproduction of *Globodera rostochiensis* (Nematoda: Heteroderidae) on indigenous South African solanaceaous plants

Introduction

Growing susceptible potatoes continuously on land infested with potato cyst nematodes (PCN) leads to heavy infestation of the soil and very small yields. *Globodera rostochiensis* (Wollenweber, 1923) Skarbilovich, 1959 only multiplies on solanaceous crops (potato, tomato and aubergine) and solanaceous weeds. Keeping the soil free of these plants for a number of years, leads to the decline in PCN populations (Whitehead & Turner, 1998). In temperate climates, the PCN populations decline by about 30% per year in the absence of a host crop (Evans & Stone, 1977). Cole & Howard, (1962b) found that 4 years of resistant potatoes have reduced the egg population to about 1% of the initial value, whereas 4 years of non-host crops have only reduced it to about 25%. In arable farming, volunteer plants can upset crop rotation and quarantine regulations and present a considerable danger. Several weeds in the genus *Solanum* and wild species of *Lycopersicon* are known to support some multiplication of PCN (van Riel & Mulder, 1998).

In order to clarify the role of root diffusates in wild potato clones resistant to PCN, Turner *et al.* (2009) conducted a series of trials using a range of PCN populations on a representation of *Solanum* species, including *Solanum sanctae-rosae, S. sparsipilum, S. gourlayi, S. acaule and S. oplocense*. Whilst a *G. rostochiensis* Ro1 population showed no variation in hatch, other

populations displayed differences in hatch in the presence of the different *Solanum* root diffusates.

Several solanaceous weeds have been observed as volunteer plants on fallow potato fields in both the Ceres and Sandveld areas. Indigenous plants of the Solanaceae also exist in the Sandveld area. In this study a range of indigenous plants and other solanaceous crops will be evaluated for their ability to sustain a population of *G. rostochiensis.* This will be the first evaluation of this kind conducted on the African continent.

Materials and methods

Obtaining cysts

Cysts of *G. rostochiensis* used in this experiment were obtained from cultures in the following way: Germinating potato tubers (cv. BP1) were disinfected with 5% household bleach for 2 minutes rinsed with water and dried. The tubers were placed in a closed container with 200 g dried sand and 30 ml distilled water. The tubers were incubated in the dark at 20°C, allowing roots to develop. Soaked, viable cysts were crushed and eggs and juveniles were added to the containers. The tubers were then incubated in the dark at 20°C. After 12 weeks new cysts were extracted using a Seinhorst cyst elutriator (Seinhorst, 1964). Cysts obtained in this manner were stored in plastic vials in the dark at 20°C for one year after harvesting to overcome diapause before using them in these experiments.

IN-EGG MORTALITY AND HATCH

Cysts in nylon bags (10 per bag) were placed in plastic pots (4 replicates), containing washed sand with the drainage holes covered with milk filters. Potato tubers (cv. BP1) and seedlings of various solanaceous plants were planted in the pots. The plant species used were *Solanum linnaenum, S. nigrum, S. tomentosum* and an unknown *Solanum* sp. that were discovered at a roadside location in the Southern Cape. *Solanum linnaenum* and *S. tomentosum* are indigenous to South Africa. The plants were maintained at a cycle of 14h light and 10h dark at 25°C in a glasshouse. The plants were removed from the glasshouse after 12 weeks and the egg content of cysts in the bags were evaluated by staining in a 0.05% aqueous solution of Meldola's blue for 1 week. The cysts were then crushed in a microcentrifuge tube with a homogeniser. The eggs and juvenile contents were released from the cysts by vortexing. The eggs were separated from the cyst fragments by washing them through a 90 µm sieve, collecting on a 25 µm sieve. Eggs and juveniles were left in a water suspension for up to 12h and observed in a counting chamber. The viability of the eggs were estimated by removing two aliquots and counting stained (non-viable) and non-stained (viable) eggs. The amount of hatched eggs was estimated by counting empty eggs. Batches of 10 cysts that were not buried in the pots were also evaluated in the same way to use as a control.

HATCHING TEST

Root exudates (PRD) of all the plants used were obtained by removing one month old seedlings from the pot and rinsing the roots to remove soil particles. The plants were then kept at 25°C with the roots in 50 ml distilled water in glass beakers for 24 h. The diffusates were then passed through a 90 µm sieve and stored at 4°C. Before use, the diffusate was

sterilised by passing through a 0.45 µm pore cellulose-acetate filter. The plant species used were potato (cv. BP1), tomato (cv. Moneymaker), *S. linnaenum, S. nigrum, S. tomentosum* and an unknown *Solanum* sp. discovered at a roadside location in the Southern Cape.

One ml of root exudate from each plant species was dispensed into the wells of a 24-well tissue culture plate. Purified water (Millipore) was used as a control. Ten cysts from each treatment (4 replicates) were placed into a well and incubated at 20°C for 4 weeks. After seven days the hatched juveniles were counted using a stereomicroscope and the cysts were transferred to new wells, this time containing fresh root exudate.

REPRODUCTIVE TESTS

Potato tubers (cv. BP1) and seedlings of tomato (cv. Moneymaker), *S. linnaenum*, *S. nigrum*, *S. tomentosum* and an unknown *Solanum* sp. that were discovered at a roadside location in the Southern Cape were planted in washed sand in plastic pots with the drainage holes covered with milk filters. Batches of cysts (4 replicates) from the hatching assay were cut open and eggs and juveniles were added to the pots. The juveniles collected from the hatching assay were also pipetted into the pots. The plants were maintained at a cycle of 14h light and 10h dark at 25°C in a glasshouse. The plants were removed from the glasshouse after 12 weeks and new cysts were extracted using a Seinhorst cyst elutriator (Seinhorst, 1964). For each container the total number of cysts was counted and the numbers of eggs estimated by counting an aliquot of the eggs removed from the cysts in a counting chamber. The multiplication rate (number of new eggs / number of eggs added originally) were calculated from the data.

DATA ANALYSIS

Data were analysed with one-way analysis of variance (ANOVA). Bonferroni multiple comparisons were used to detect differences among the main effects. In all tests a significance level of 5% was used. Statistical analyses were carried out using STATISTICA version 11 (StatSoft Inc. 2012).

Results

ESTIMATION OF IN-EGG MORTALITY AND HATCH

The changes occurring in the cyst contents when buried with different plants for 12 weeks are shown in Figure 12.1. Data analysed with a one-way ANOVA showed significant differences ($F_{(5, 18)} = 11.010$; $P = 0.00006$) when comparing the percentage of viable eggs per cyst. A significant decrease ($P < 0.05$) was observed in the percentage of viable eggs per cyst with the potato (2.4% ± 2.6% viable eggs), *S. nigrum* (14.5% ± 9.8% viable eggs) and the unknown *Solanum* species (19.0% ± 14.6% viable eggs) treatments when compared to the control (45.5% ± 5.4% viable eggs), but not with *S. tomentosum* and *S. linnaeum.* Data analysed with a one-way ANOVA also showed significant differences ($F_{(5, 18)} = 12.820$; $P = 0.00002$) when comparing the percentage of hatched eggs per cyst. The percentage of hatched eggs increased significantly ($P < 0.05$) for the potato (81.8 ± 4.1 % hatched eggs) and *S. nigrum* (68.9% ± 10.1% hatched eggs) treatments when compared to the control (34.9% ± 5.9% hatched eggs). An increase was also observed with the unknown *Solanum*, albeit not

significant. Data for the percentage of dead eggs in the cysts showed no significant differences when analysed with a one-way ANOVA ($F_{(5, 18)} = 1.5385$; $P = 0.22761$).

HATCHING TEST

Figure 12.2 shows the cumulative emergence of juveniles from cysts incubated in root exudates from the different plants. Data of the total hatch as measured at week 4 showed significant differences when analysed with one-way ANOVA ($F_{(6, 21)} = 11.366$; $P = 0.00001$). Cysts incubated in the tomato root exudate had the highest number of juveniles that hatched from the cysts (342 ± 76), followed by potato root exudate (172 ± 66) and *S. linnaeum* root exudate (136 ± 80). Only tomato root exudate and potato root exudate caused a significant ($P < 0.05$) increase in the amount of juveniles that hatched, when compared to the water control. The amount of hatch from cysts incubated in the other root exudates did not differ significantly from those in the water control.

REPRODUCTIVE TESTS

The results of the reproductive test are shown in Table 12.1. Multiplication rates on the potato and tomato were higher than one, but those on the solanaceous weeds were either zero or very low.

Discussion

Differential results from the viability staining and hatching test could not confirm that any of the solanaceous weeds tested, induce substantial hatch in *G. rostochiensis.* Potato and tomato plants induced substantial hatch in *G. rostochiensis*, as reflected by the significant increase in hatched eggs and J2 emergence that was observed in the viability and hatching tests. None of the plants tested caused mortality of eggs in the cysts. Reproduction tests confirmed tomato and potato as hosts and suggests that the solanaceous weeds do not support multiplication of *G. rostochiensis*, but very low numbers of the nematode might be able to survive on these plants.

In South Africa, seed potatoes are only to be planted on infested fields after 8 years of non-host cultivation and only if an official test shows it to be free from *G. rostochiensis*. The control of solanaceous weeds on these plots must be considered as an added measure to eradicate *G. rostochiensis* in these plots.

Literature

COLE, C.S. & HOWARD, H.W. (1962) The effect of growing resistant potatoes on a pota-root eelworm population – a microplot experiment. *Annals of Applied Biology* 50, 121-127.

EVANS, K. & STONE, A.R. (1977). A review of the distribution and biology of the potato cyst nematodes *Globodera rostochiensis* and *G. pallida*. PANS 23, 178-189.

SEINHORST, J.W. (1964). Methods for the extraction of *Heterodera* cysts from not previously dried soil samples. *Nematologica* 10, 87–94.

STATSOFT, INC. (2012). STATISTICA (data analysis software system), version 11. www.statsoft.com.

TURNER, S.J., FLEMING, C. C., MORELAND, B. P. & MARTIN, T.J.G. (2009). Variation in hatch among pathotypes of the potato cyst nematodes, *Globodera rostochiensis* and *G. pallida*, in response to potato root diffusates from *Solanum* spp. I. Preliminary assessments to establish optimal testing conditions. *Nematology* 11, 749-756.

VAN RIEL, H.R. & MULDER, A. (1998). Potato cyst nematodes (*Globodera* species) in Western Europe. In: Marks, R.J. & Brodie, B.B. (Eds.). *Potato Cyst Nematodes. Biology, distribution and control.* CAB International, Wallingford, Oxon OX10 8DE, UK, pp. 135-152.

WHITEHEAD, A.G. & TURNER, S. J. (1998). Management and regulatory control strategies for potato cyst nematodes (*Globodera rostochiensis* and *Globodera pallida*). In: Marks, R.J. & Brodie, B.B. (Eds.). *Potato Cyst Nematodes. Biology, distribution and control.* CAB International, Wallingford, Oxon OX10 8DE, UK, pp. 135-152.

Table 12.1: Reproduction of different populations of *Globodera rostochiensis* on different solanaceous plants.

Plant species	Multiplication
Potato (cv.BP1)	7.425
Tomato (cv. Moneymaker)	2.75
Solanum linnaenum	0.025
Solanum nigrum	0.000
Solanum tomentosum	0.125
Solanum sp.	0.000

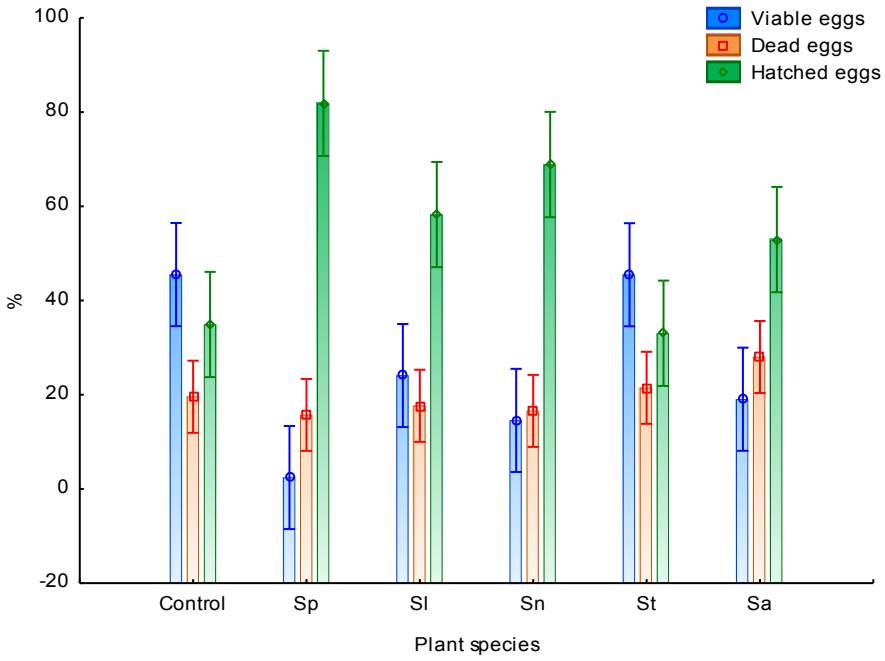

Fig. 12.1: Viability of *Globodera rostochiensis* eggs from cysts buried for 12 weeks in pots with different solanaceaous plants. C = control, Sp = potato (cv. BP1), Sl = *Solanum linnaeum*, Sn = *Solanum nigrum*, St = *Solanum tomentosum* and Sa = unknown *Solanum* sp. The top of the vertical bars denote the means and the lines upwards and downwards the 95% confidence intervals for the true means.

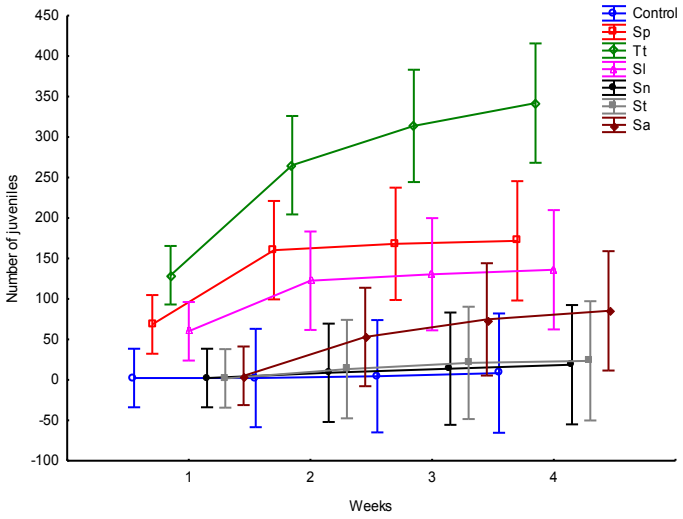

Fig. 12.2. Cumulative hatch from *Globodera rostochiensis* cysts incubated in root exudates from different solanaceous plants. C = control, Sp = potato (cv. BP1), Tt = Tomato (cv. Moneymaker), Sl = *Solanum linnaenum*, Sn = *Solanum nigrum*, St = *Solanum tomentosum* and Sa = unknown *Solanum* sp. Vertical bars denote 95% confidence intervals for the true means.

Chapter 13

Assessing the reproductive ability of *Globodera capensis* (Nematoda: Heteroderidae) on potato

Introduction

Potato cyst nematodes (PCN) have been recorded on the African continent from Libya, Algeria, Morocco, Tunisia, Egypt, Sierra Leone, Mozambique and South Africa (Kleynhans, 1998). No indigenous *Globodera* spp. has been found in Africa until the recent discovery of such a species in the Sandveld region on the West Coast of South Africa. This cyst nematode has since been described as *Globodera capensis* Knoetze, Swart & Tiedt, 2013. *Globodera capensis* has been found on several farms in the Swartland and Sandveld areas in South Africa. The cysts of the new species vary from turbiniform, subglobose to spherical in form and from yellow to dark brown in colour. *G. capensis* are morphologically and morphometrically very near or indistinguishable from PCN (*G. rostochiensis* and *G. pallida*), *G. artemisiae*, *G. millifolii* and especially *G. tabacum tabacum*, one of the three subspecies of *G. tabacum*. It can, however, be distinguished from these species by the molecular characteristics of the ITS-region of the ribosomal DNA (Knoetze *et al.*, 2006).

The presence of *G. capensis* in the Sandveld region has complicated the detection of the quarantine nematode, *G. rostochiensis* in regulatory samples from this area, since they are present in most samples and are morphologically very similar to *G. rostochiensis.* Exporters of potatoes need to have their fields tested for the presence of *Globodera* spp. The South African Seed Potato Certification Scheme also requires plots to be free of *Globodera* spp. It

is therefore important to determine if this nematode is a pest of potato, since the knowledge will impact heavily on accessibility to foreign markets and certification of seed potatoes. In this study, the reproductive ability of *G. capensis* is evaluated in the laboratory, glasshouse and in the field.

Materials and methods

LABORATORY TESTS

Origin of cysts

Sandy soils were collected from six different plots in the Sandveld region of South Africa, known to contain *Globodera* cysts (Table 13.1). Cysts were extracted using the Seinhorst cyst elutriator (Seinhorst, 1964). Although a higher number of cysts were subjected to the hatching test, many turned out to contain no eggs or larvae upon examination after the completion of the test.

Hatching test

Potato root exudate was obtained by pouring water through pots containing potato plants and collecting excess water that trickles out of the pots. The exudate was stored at 4°C. The nematode cysts were soaked overnight in distilled water after extraction and then transferred to a syracuse dish containing potato root exudate. Only one cyst was allocated to each Syracuse dish. The cysts were incubated at 20°C for 4 weeks. The dishes were checked weekly with a stereo microscope for signs of hatching. After each check the potato root

exudate in each dish was replaced with fresh exudate. After 4 weeks cysts that have not hatched yet were cut open to determine if they contained any eggs or larvae. Cysts of *G. rostochiensis* were used as positive control and received the same treatment as above.

Reproductive test

The viable cysts from the hatching tests were also inoculated to determine their reproductive ability on potatoes. Germinating potato tubers (cv. BP1) were disinfected with 5% household bleach for 2 min, rinsed with water and dried. The tubers were placed in a closed container with 200 g dried clean sand and 30 ml distilled water. The viable cysts were crushed in a microcentrifuge tube with a homogeniser and then added to the sand. The tubers were then incubated in the dark at 20°C for 14 weeks and inspected for the presence of cysts.

GLASSHOUSE TESTS

Origin of cysts

Cysts were extracted from samples collected for regulatory purposes from various plots in the Sandveld region, using the Seinhorst cyst elutriator (Seinhorst, 1964). After extraction cysts were handpicked from filter papers and stored in microcentrifuge tubes at 4°C until further use. Table 13.2 lists the plots in the Sandveld area from which cysts were obtained.

Reproductive test

Forty cysts of *G. rostochiensis* as well *G. capensis* (4 repetitions, 10 cysts per repetition for each species of cyst) were soaked in distilled water for three days. The water was removed and potato root diffusate was added to each watch glass containing cysts and incubated for 7 days at 25°C. Potato tubers (cv. VanderPlank) were planted in washed sand in plastic pots with the drainage holes covered with milk filters. The cysts were crushed in a microcentrifuge tube with a homogeniser and eggs and juveniles were added to the pots (4 repetitions, 10 cysts per repetition for each species of cyst). The plants were maintained at a cycle of 14h light and 10h dark at 25°C in a glasshouse. The plants were removed from the glasshouse after 12 weeks, after which the number of new cysts were determined. The mean multiplication rates of the populations were calculated and expressed as P_f / P_i, where P_f = initial cyst population and P_i = final cyst population.

Viability of the cysts used

Ten cysts each of *G. rostochiensis* and *G. capensis* were used to determine the viability of the inoculum used in the reproductive test. Soaked cysts were placed in 0.05% aqueous solution of Meldola's blue for 1 week. The stain was removed by rinsing the cysts well in water and then releasing the eggs by crushing in a microcentrifuge tube with a homogeniser. The eggs were separated from the fragments by washing them through a small sieve. Eggs and juveniles were left in a water suspension for up to 12h and observed in a counting chamber. The percentage of viable eggs were estimated by removing two aliquots and counting stained (non-viable) and non-stained (viable) eggs. Spontaneous hatch was estimated by counting hatched (empty) eggs.

FIELD TRIAL

Trial site

A 25 hectare potato field, PWK2, located on the farm Sandberg in the Sandveld region (32° 19.701'S; 18° 32.904'E) was selected for this trial. Upon commencement of the trial the field has been left fallow for 5.5 years. Routine sampling in October 2009 revealed a viable population of *G. capensis* in this field. The field was also sampled in October 2011 to confirm the presence of the nematode. At this stage the field was found to contain an average of 7.75 cysts 500 cc soil^{-1}. An agreement was made with the farmer to monitor the levels of *G. capensis* during the next production cycle on this field. For this purpose, a special order was issued by the Directorate: Inspection Services, allowing the production of table potatoes on the field in the 2012 growing season. Planting was scheduled for February 2012, with harvest projected for August 2012. The field, consisting of very sandy soil, was planted with potatoes (cv. Avalanche) and irrigated from a central pivot. The Sandveld area is part of a warmer temperate zone, with very light frost.

Sampling

The field was sampled before planting (19/01/12), during the growing season (29/05/12) and after harvest of the crop (20/09/12)). On each occasion four samples were obtained from the field by taking 50 core samples, distributed systematically over a maximum area of 5 ha. To ensure systematic distribution of core-samples, sampling was done in a zigzag pattern across the field. Core-samples were obtained by using a soil sampling auger of the “cheese-sampler” type with half-cylindrical blade 20-30 cm long and 20-25 mm wide. Sampling was done to a

depth of approximately 10 cm, resulting in a final sample of approximately 2000 cc. At each sampling date, at least 10 plants (potatoes as well as opportunistic plants) were removed and inspected microscopically for the presence of cysts and females.

*Recovery of cysts and viability of eggs*Cysts were extracted using the Seinhorst cyst elutriator (Seinhorst, 1964) from the entire sample of approximately 2000 cc soil. Soaked cysts were placed in 0.05% aqueous solution of Meldola's blue for 1 week. The stain was removed by rinsing the cysts in water and then releasing the eggs by crushing with a homogeniser in a microcentrifuge tube. The eggs were separated from the fragments by washing them through a small sieve. Eggs and juveniles were left in a water suspension for up to 12h and observed in a counting chamber. The percentage of viable eggs were estimated by counting stained (non-viable) and non-stained (viable) eggs. Hatch was estimated by counting empty eggs. The identity of the cysts was confirmed by PCR amplification of the ITS1 region and restriction digest with A*lu*I.

Results

LABORATORY TESTS

Hatching test and reproduction

Viable cysts from three plots showed no signs of hatching. No viable cysts were found in one of the plots. All the viable cysts tested from the Nuwerus plots hatched while 50% of the cysts from the Wadrif plot showed signs of hatching. It must, however, be mentioned that the

amount of larvae emerging from these cysts were low (2 or less). Table 13.3 shows the amount of cysts that hatched. All the cysts from the positive controls hatched.*t*

All the cysts that did not hatch in potato root exudate did not reproduce on the tubers (See Table 13.3). No cysts could be found after 14 weeks on these cultures. Cysts from the positive control and the Nuwerus population (identified as *G. rostochiensis*) did reproduce, having 2 to 6 viable cysts on the roots and in the sand of the culture. Five of the inoculations from the Nuwerus cysts could not be used due to premature rotting of the potatoes, but the remaining three did show reproduction of the nematode.

GLASSHOUSE TESTS

Reproductive test

The results for this experiment are shown in Table 13.4. On all the plants inoculated with *G. rostochiensis*, reproduction occurred successfully (Average multiplication rate = 21.8). No new cysts could be found on any of the plants inoculated with *G. capensis*.

Viability test

The results of viability staining are shown in Table 13.5. The batch of *G. rostochiensis* cysts contained an average of 83 viable eggs per cyst, and the batch of the unknown *Globodera sp.* contained an average of 42 viable eggs per cyst.

FIELD TRIAL

*Recovery of cysts and viability of eggs*Low amounts of cysts were extracted from all the samples taken at the trial site. Table 13.6 shows the amounts of cysts extracted from all the samples at each sampling date. No cysts or females were observed on any of the plants lifted at each sampling date.

Egg viability

Results are expressed as hatched eggsl, dead eggs and viable eggs per 500 cc soil (Fig. 14.1). Regression analysis showed that viable eggs 500 cc^{-1} soil were the only dataset fitted by a linear relationship with time with a coefficient of linearity (R^2) of 97.6%. The overall decline rate based on viable eggs 500 cc^{-1} soil was 36.05% and the multiplication factor was 0.64.

Discussion

PCR-RFLP (Knoetze *et al.*, 2006) confirmed the identity of cysts used in the laboratory and glasshouse tests as either *G. rostochiensis* or *G. capensis*. The identity of the cysts that did not hatch in experiment A was determined as that of *G. capensis*. The cysts from the Nuwerus population (all hatched) in experiment A were identified as *G. rostochiensis.* The three cysts from the Wadrif population that showed signs of hatching were identified as *G. capensis*. The cysts that showed signs of hatching were probably the result of spontaneous hatching which has been recorded in other *Globodera* spp.

The results from the reproductive test in the laboratory tests show that *G. capensis* was unable to reproduce on potatoes under laboratory conditions. In the glasshouse tests, the inoculum from *G. capensis* were determined as viable by staining of a representative batch of the cysts, but still no reproduction on potato was observed under glasshouse conditions. In both experiments, the *G. rostochiensis* control was able to reproduce successfully on the potato plants from the same cultivars as were used for the unknown cysts.

PCR-RFLP confirmed the identity of all cysts detected from the field trial as *G. capensis.* Although the number of cysts detected declined over the period, standard deviation over the different samples are too great to consider this significant. Regression analysis of the average viable eggs 500 cc^{-1} soil shows a negative correlation over time during the observed period, resulting in a decline rate of 36%. If we also consider the multiplication factor of <1, there is no evidence that *G. capensis* was able to reproduce on potato (cv. Avalanche) under field conditions.

In conclusion, viable cysts of *G. capensis* were unable to reproduce on potato plants (cv. BP1, VanderPlank and Avalanche) under laboratory, glasshouse and field conditions.

Literature

KLEYNHANS, K.P.N. (1998). Potato cyst nematodes (*Globodera* species) in Africa. In: Marks, R.J. & Brodie, B.B. (Eds.). *Potato Cyst Nematodes. Biology, Distribution and Control.* CAB International, Wallingford, Oxon OX10 8DE, UK, pp. 347-351.

KNOETZE, R., MALAN, A.P. & MOUTON, C. (2006). Differentiation of South African potato cyst nematodes (PCN) by analysis of the rDNA internal transcribed spacer region. *African Plant Protection* 12, 103–110.

KNOETZE, R., SWART, A. & TIEDT, L.R. (2013). Description of *Globodera capensis* n. sp. (Nematoda: Heteroderidae) from South Africa. *Nematology* 15, 233-250.

SEINHORST, J. W. (1964). Methods for the extraction of *Heterodera* cysts from not previously dried soil samples. *Nematologica* 10, 87–94.

Table 13.1. Origin of cysts used in laboratory tests to determine the reproductive ability of *Globodera capensis* on potato.

Farm	Amount of cysts used	Amount of viable cysts
Taaiboschkraal	2	0
Rhenosterfontein	11	5
Wadrift	14	1
Bottelfontein	21	5
Nuwerus	10	8
Wadrif	15	6

Table 13. 2. Origin of cysts used in glasshouse tests to determine the reproductive ability of *Globodera capensis* on potato.

Farm	Plot	Date sampled
Sandberg	PWK	21/10/09
Wadrift	H1	16/10/09
Modderfontein	Sirkel 12	15/10/09
Rhenosterfontein	RA	14/10/09
Kruispad	Charlieskraal	15/10/09
Nupost	N4	16/10/09

Table 13.3. Results from laboratory tests to determine the reproductive ability of *Globodera capensis* on potato.

Origin	Viable cysts	Amount hatched	Reproduction on potato
Positive control	5	5	Yes
Taaiboschkraal	0	0	No
Rhenosterfontein	5	0	No
Wadrift	1	0	No
Bottelfontein	5	0	No
Nuwerus	8	8	Yes
Wadrif	6	3	No

Table 13.4. Results from reproductive test (glasshouse) to determine the reproductive ability of *Globodera capensis* on potato.

Repetition	Inoculum	New cysts detected	Multiplication rate
R1	*G. rostochiensis*	265	26.5
R2	*G. rostochiensis*	92	9.2
R3	*G. rostochiensis*	296	29.6
R4	*G. rostochiensis*	218	21.8
C1	*G. capensis*	0	0
C2	*G. capensis*	0	0
C3	*G. capensis.*	0	0
C4	*G. capensis*	0	0

Table 13.5. Results from viability staining to determine the reproductive ability of *Globodera capensis* on potato.

	Average hatched eggs / cyst	Average non-viable eggs / cyst	Average viable eggs / cyst
G. rostochiensis	97	35	83
Unknown *Globodera*	61	22	42

Table 13.6. Cysts recovered from samples collected at plot PWK2 during the 2012 growing season.

Sampling event	Cysts 500 cc^{-1}					
	Sample 1	Sample 2	Sample 3	Sample 4	Average	Std. dev.
Before plant	4.25	3.25	0.75	2.75	2.75	1.27
During growing	3.00	2.00	4.00	1.50	2.63	0.96
After harvest	2.25	0.75	0.33	3.50	1.71	1.26

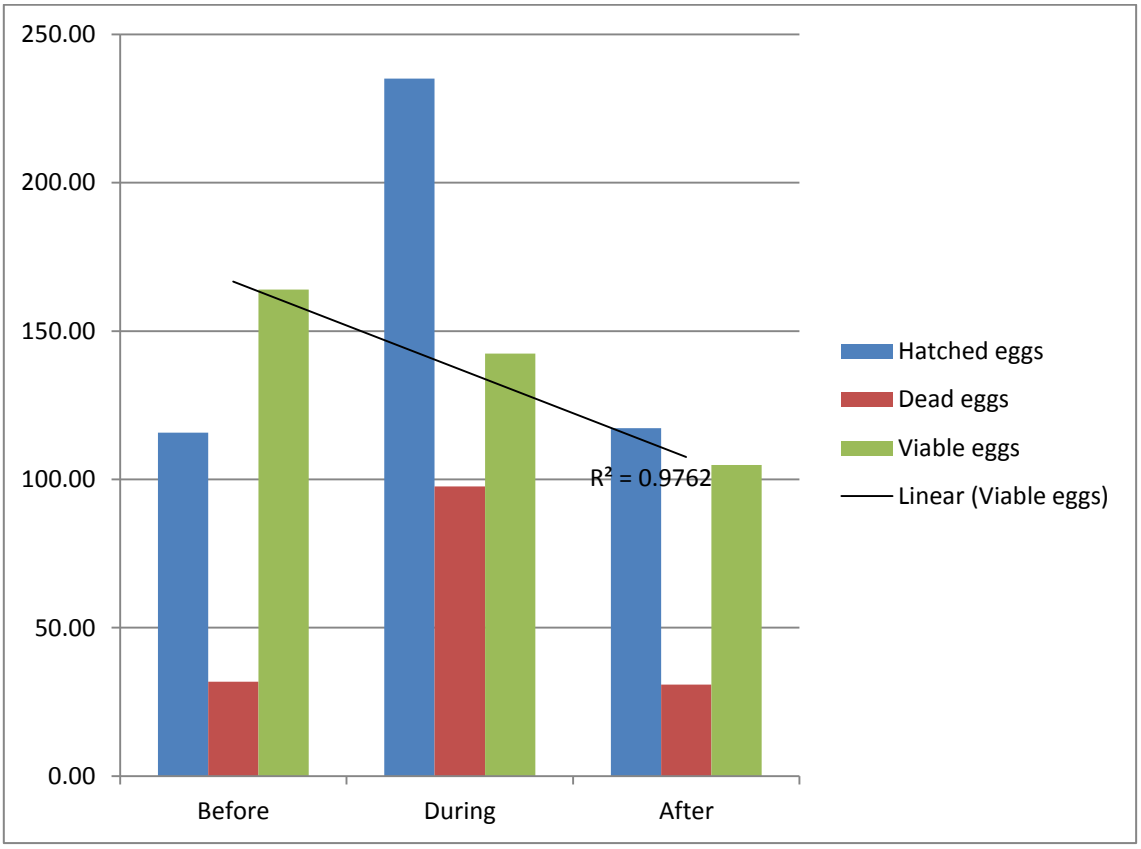

Fig. 13.1: Average amount of eggs 500 cc^{-1} soil of *Globodera capensis* in a potato field during one growing season with a linear trendline for viable eggs inserted.

Chapter 14

Conclusions

The aim of this study was to characterise the genus *Globodera* in South Africa and to contribute towards the global knowledge of the biodiversity, systematics and biology of the group. Specifically, it was aimed to determine the spread of the genus in South Africa; to study the systematics and describe the characteristics of the group in South Africa and to gain a more complete understanding of the biology of the group as agricultural pests, enabling nematologists to make informed decisions for the management of these nematodes.

The survey of the Cape Floristic Region of South Africa constitutes the first survey specifically targeted at the detection of indigenous cyst nematodes in South Africa. This survey unearthed four new species of cyst nematodes, whose morphological and molecular characteristics may yet prove to be of great value for the study of the evolution and biogeography of the group. Future surveys in other regions of South Africa might lead to the discovery of more cyst specimens for study. Since the host plants of the cyst nematodes that have been discovered is not known, further research into this aspect is warranted.

A countrywide survey in the potato-producing areas of South Africa enabled us to determine the spread of potato cyst nematodes in these areas. The survey confirmed the presence of *Globodera rostochiensis* in four areas and detected the presence of *G. rostochiensis* in another area, the South-Western Cape, while it also confirmed the absence of *G. pallida* from all production areas. Analysis of survey results allowed DAFF to identify shortcomings in current survey protocols and make certain recommendations for future surveys and for the

management of this quarantine nematode from a regulatory perspective. *G. rostochiensis* poses a significant threat to the potato industry in South Africa and the distribution of the nematode to new potato-producing areas must be prevented at all costs. The analysis of molecular sequence data established the distinct phylogenetic positions of four cyst nematode populations from South Africa, relative to an array of other cyst nematode species. Characterisation and phylogenetic analyses of sequences from three populations confirmed that they belong to the genus *Globodera* and indicated that they are indeed unique species, previously undescribed. According to the phylogenetic analyses another population of cyst nematodes (herein referred to as population WK2) did not belong to *Globodera*. Studies of phylogenetic relationships among these nematodes are not only essential to taxonomy, but also allow a more complete understanding of the evolution of the group. The discovery of new *Globodera* species in Southern Africa supports the theory of a Gondwanaland origin of the genus and the theory that divergence of the two main *Globodera* lineages might have occurred subsequent to the break-up of Africa and South America. Furthermore, the results of molecular analyses contribute to our estimation of biodiversity through the systematic addition of more species, more isolates and extensive collateral data to sequence databases.

The provision of molecular protocols for the accurate identification of these nematodes enable greater accuracy in the identification of cyst nematodes in South Africa, which are required for the economic and regulatory management of these nematodes. Problems encountered with the design of species-specific primers to distinguish indigenous *Globodera* spp. from *G. rostochiensis* in samples from potato fields, highlighted the fact that the development of new molecular tools should include an obligatory validation process on a set of populations, including non-agriculturally important *Globodera* spp., before adoption as a reference test. Although the digestion of the ITS region of rDNA with selected restriction

enzymes, proved to be a useful diagnostic test for the discrimination of *Globodera* spp., the amplification of the ITS regions of rDNA, followed by direct sequencing of the PCR product is recommended. Future research in this regard should include the exploration of other gene regions for discrimination of the different species. Possible regions that could be utilised include the Hsp90 gene and the chorismate mutase gene.

The study of the morphology of three indigenous populations of cyst nematodes confirmed the results of the molecular study, which lend support to the theory that they are new species of *Globodera*. A combination of the molecular, morphological and morphometric characteristics of these populations were used to describe them as new *Globodera* species The description of these species and the development of reliable diagnostic methods for their discrimination is of great importance to growers and regulatory services alike, because of the complications in the detection of the quarantine nematode, *G. rostochiensis* that could arise due to the presence of these cysts in regulatory samples from potato-producing areas. Since the possible emergence of a new *Globodera* sp. parasitizing potato would impact heavily on accessibility to foreign markets and certification of seed potatoes in South Africa, it was important to determine if *Globodera capensis*, which are often found on fallow potato fields, is a pest of potato. The reproductive ability of *G. capensis* on potato was therefore evaluated in the laboratory, glasshouse and in the field and the conclusion was that viable cysts of *G. capensis* were unable to reproduce on potato plants under these conditions.

Morphological analysis of the WK2 population, indicated by molecular data as not belonging to *Globodera*, revealed a unique labial morphology , as well as the unusual presence of a vulva and functional gonads in some of the “juvenile” specimens, which set it apart from any known genus of the Heteroderidae. Further studies on this unique cyst nematode may prove to reveal exciting new insights into the knowledge of the origins, evolution and

phylogeography of the cyst nematodes as a group, but caution should be exercised, because of the small amount of specimens available for study.

Analysis of data from RAPD banding patterns, revealed intraspecific genetic variation between populations *G. rostochiensis*, especially a population from the Sandveld that was genetically different from the other populations within the species. However, these genetic differences was not expressed in the morphological features, leading to the usage of other methods for the determination of intraspecific variations between these populations, including pathotype tests, hatching tests and reproduction tests. Further information about the origins, genetic diversity and its linkage with geographic location of these populations can be deducted from RAPD studies, if more local isolates together with isolates from other continents are studied in the same manner.

Experiments to determine the effect of storage temperature on the viability and hatching of South African populations of *G. rostochiensis*, showed differences in the responses of different populations to different storage temperatures w.r.t. hatching and viability of eggs in the cysts, especially a population from the Eastern Cape, which survived better at higher temperatures than any of the other populations. These results also suggest that storage of cysts at higher temperatures does not directly influence the hatching ability of eggs, but that hatch is decreased because of the increased mortality of eggs in the cyst. The burial of cysts in different locations for two years caused a decline in viable eggs in cysts from different populations, but suggests that the cysts will be able to survive for much longer in these soils. This observation was confirmed by observation of infested plots where viable cysts remain in the soil after more than 3 years of the plots being fallow. Since no significant increase in the amount of dead eggs was observed, spontaneous hatch is deemed to be the main contributor to the decline of viability of these cysts in the time period of the study.Future research into

the survival of the cysts for periods longer than two years are needed to determine the appropriate quarantine period on infected fields for local conditions.

Recording of soil temperatures in two locations have shown that the average temperature at 20 cm depth is not as high as was believed at the beginning of this study. These measurements, together with the results from hatching and viability tests suggest that *G. rostochiensis* might be able to survive without a host for longer periods in the extreme climates of the Sandveld and Ceres areas, than was previously believed. Suggestions that the quarantine period for infested plots in these areas can be shortened were therefore premature and unsubstantiated. The results of experiments to assess the *in vitro* reproduction ability of these populations confirmed that the optimum temperature for the reproduction of *G. rostochiensis* is around 20°C, but that multiplication and survival is influenced negatively when the temperatures rise above 25°C. As the average temperature of soils in two measured "hot" regions is around 20°C, we can safely say that the reproduction of *G. rostochiensis* will not be negatively influenced by temperature in these regions, an assumption that is supported by high crop losses in some affected areas. Investigation into the occurence of a second generation in one cropping season, as was previously observed in other warm regions, should be considered when future research into the life cycle of *G. rostochiensis* under local conditions are planned.

Reproduction on differential potato clones confirmed the pathotype of all the South African populations of *G. rostochiensis* as Ro1, a key factor of value for management of the nematode and breeding programmes for resistant potato cultivars. After assessing the reproduction of *G. rostochiensis* on indigenous solanaceous plants, it was concluded that none of these plants, induce substantial hatch in *G. rostochiensis*, nor do they support multiplication of the nematode. It was concluded that very low numbers of the nematode

might be able to survive on these plants, a factor that should be considered when eradication of the nematode is targeted by non-host cultivation on infected plots. This was the first evaluation of this kind conducted on the African continent.

In conclusion, the aims of the study were successfully met. Knowledge of the distribution and systematics of *Globodera* spp. in South Africa has been greatly enhanced through this study and we have gained insightful knowledge of the biology of this group, and its importance in the management and regulation of these nematodes.

Scientia est potentia